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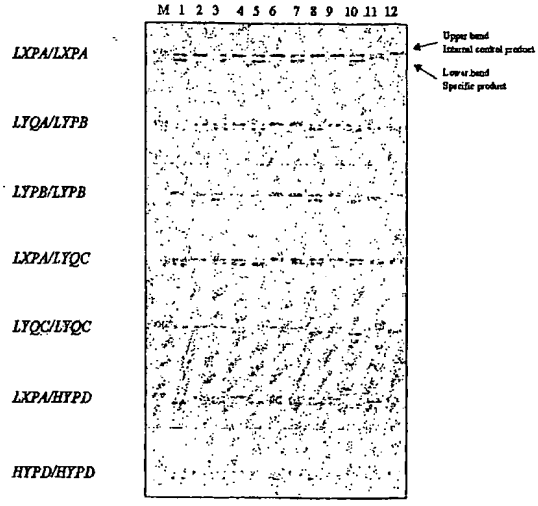
(54) Title: A METHOD OF SEPSIS PROGNOSIS



(57) Abstract: The present invention relates to a method of predicting whether an individual having Systemic Inflammatory Response Syndrome (SIRS) will develop sepsis by correlating the MBL genotype with a predefined risk value associated with said particular MBL genotype. A predefined risk value associated with aid particular MBL genotype in the present context can be divided in a high risk and a low risk for developing sepsis in said individual having SIRS. Typically, a high risk relates to a MBL genotype that is characterised in having at least one variant structural allele of the MBL gene and/or having two low-expression regulatory alleles of the MBL gene in said samples.

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A method of sepsis prognosis

Field of invention

The present invention relates to a method for prognostic purposes of estimating whether an individual will develop sepsis when said individual suffers from systemic inflammatory response syndrome (SIRS).

Background of invention

The systemic inflammatory response syndrome (SIRS) is associated with different overlapping scenarios comprising invasive infection, dissemination of microbes secondary to injury, shock and activation of inflammation by apparent non-infectious events. SIRS can be self-limited or can in infected patient's progress to severe sepsis and septic shock. Severe sepsis is a primary cause of death in intensive care units (ICU) ranging from 30 to 50 percent.

Failure of host defence mechanisms is likely to be involved in the variable presentation of SIRS and sepsis. Although the pathophysiology of SIRS and sepsis is very complex, it has been shown that genetic factors of importance for the inflammatory response such as polymorphism's in the promoter region of the tumour necrosis factor alpha (TNF-alpha) gene may be associated with susceptibility and outcome of septic shock. In addition, genetic variation within the fibrinolytic system seems to be of importance for the outcome of sepsis (Menges et al. 2001).

Mannose-binding lectin (MBL) is a circulating liver synthesised serum protein of importance for innate immune defence and is one of the recognition molecules in the lectin complement activation pathway. Human MBL is derived from a single gene located on chromosome 10 (*mbl2*). Inter-individual differences in MBL serum concentrations are caused by structural alleles (*B*, *C* and *D* = codons 54, 57 and 52, respectively) in the *mbl2* gene. The normal allele is named *A* and the common designation for the variant alleles is *0*. In addition to structural allele variants, the differences in MBL serum levels are determined by polymorphic sites in the promoter region of the *mbl2* gene . Particularly, a base substitution in codon -221 (G to C) (promoter allele *X*) is associated with low MBL serum concentration (Madsen HO. et al. 1995 and Madsen HO. et al. 1998).

MBL deficiency is associated with increased risk of infections during early childhood, especially in ages between 6 and 18 months and also in patients with a concomitant immunodeficiency. In addition several studies have suggested, that MBL variant alleles may be weakly associated with autoimmune conditions such as systemic lupus

erythematosus as well as progression of rheumatoid arthritis. However, the importance of MBL as susceptibility and modifying factor for development of sepsis is unknown.

WO 02/05833 in general relates to the use of sub-units and oligomers of MBL in prophylactic and/or curative treatment of infections in an individual receiving TNF-inhibiting treatment. The description further describes an aspect of using a measurement of the MBL level as a prognostic marker for the risk of the individual receiving TNF-inhibiting treatment of acquiring an infection, and thereby an indication of the need for treatment. All patients are selected among individuals that have received treatment with either etanercept or infliximab and presented clinically significant infections.

WO 00/70043 relates to a process of producing a recombinant human MBL composition, a recombinant human MBL composition, a gene expression construct encoding a recombinant MBL polypeptide and to use of a MBL composition for the production of a pharmaceutical composition for the treatment, in an individual, of clinical conditions selected from infections, MBL deficiency, cancer, disorders associated with chemotherapy, miscarriages, disorders associated with neutropenia, and human immunodeficiency virus (HIV).

US 2001/0014449 relates to methods for the analysis and determination of the nature of single nucleic acid polymorphism's (SNPs) in a genetic target. The nature of the SNPs is determined by the steps of providing a plurality of hybridisation complexes arrayed on a plurality of test sites on an electronically bioactive microchip. The MBL gene locus is mentioned as a useful target since there are multiple closely spaced SNPs at a single genetic locus. MBL gene locus is also described as correlating with susceptibility to sepsis in leukopenic patients. US 2001/0014449 does not relate to the susceptibility to sepsis in SIRS patients.

MBL genotypes and serum concentrations have previously been determined in unrelated ethnic populations and that high frequencies of two different variant alleles are associated with low MBL concentrations (Madsen H.O. et al. 1998).

Mullighan suggest MBL gene polymorphism's are associated with major infection following allogenic hemopoietic stem cell transplantation (Mulligahn C.G. et al.), Steffensen describes the distribution of some variants of the MBL gene in a population of healthy unrelated Danes and correlates the genotype with the plasma MBL concentrations (Steffensen R. et al.) and others suggest the plasma level of MBL as predictor of sepsis after chemotherapy (Theil et al. 2000), thus limiting these conclusions to such subjects.

Thus, the importance of MBL in particular as susceptibility and modifying factor for development of sepsis in a patient having SIRS is unknown.

Summary of invention

The present inventors have found a method of predicting whether and to what extend there is a risk of an individual having SIRS will develop sepsis based on the genotype and/or phenotype of said individual, and/or on the MBL concentration, in a biological sample of said individual.

The estimation of the sepsis risk of an individual can involve the comparison of the number and/or kind of polymorphic sequences of an individual and/or the MBL protein concentration in a biological sample of an individual with a predetermined sepsis risk profile. Such a profile can be based on statistical data obtained for a relevant reference group of individuals.

In one aspect the present invention concerns a method of predicting whether an individual having SIRS will develop sepsis, said method comprising the steps of:

a) providing a biological sample from said individual,

b) determining the mannose-binding lectin (MBL) genotype of said individual,

c) predicting the risk of said individual of developing sepsis by correlating the MBL genotype of step b) with a predefined risk value associated with said particular MBL genotype.

In another aspect the invention relates to a method of predicting whether an individual will develop sepsis comprising the steps of:

a) providing a biological sample from said individual,

b) determining the MBL concentration in said individual,

c) predicting the risk of said individual of developing sepsis by correlating the MBL concentration of step b) with a predefined risk value associated with said particular MBL concentration.

The invention also describes a kit for predicting whether an individual will develop sepsis.

Detailed description of the invention

The genomic method

Many factors are involved in controlling and limiting localised infections. In general, septic response occurs when immune defences fail to contain an invading microbe. Many cases of sepsis are triggered by microbes that do not ordinarily cause systemic disease. The present invention discloses the connection between the MBL genotype of an individual having SIRS and the risk of developing sepsis, severe sepsis or septic shock.

The invention discloses how decreased levels of MBL and lack of functional MBL is crucial to the development of sepsis and septic shock in an individual having SIRS. Steps prior to an actual diagnosis of SIRS in a patient are furthermore an object of embodiments of the present invention, and the skilled addressee would easily recognise that employing the present invention on patients with diseases which are likely to evolve to SIRS, such as but not limited to infections, especially bacterial infections, traumas, tissue damages and burns; evaluating the genotype and/or concentration of MBL; and applying, for instance, MBL to said patient can prevent fatal outcome for those patients having high risk of later developing sepsis, as described by the present invention. Thus, it should be understood that any feature and/or aspect discussed above in connection with SIRS according to the invention apply by analogy to such prior stages of disease according to the invention.

Genetic factors may predispose to increased risk for sepsis in critically ill patients. Mannose-binding lectin (MBL) is an important factor in innate immune defence, and the present application discloses that MBL gene polymorphism's causing low levels of MBL are associated with the development and progression of sepsis in adult intensive care patients.

272 patients with systemic inflammatory response syndrome (SIRS) were followed prospectively for different MBL genotypes and compared by the present inventors with respect to microbiology, sepsis development, and survival.

The comparison shows that presence of MBL variant alleles is associated with development of sepsis, severe sepsis and septic shock.

An increased risk of death is observed in variant allele carriers. These data show that a genetic factor as MBL insufficiency plays an important role in the susceptibility of critically ill patients for development and progression of sepsis and confer a substantial risk for fatal outcome.

Accordingly, the present invention relates to a method of predicting whether an individual having Systemic Inflammatory Response Syndrome (SIRS) will develop sepsis comprising the steps of:

a) providing a biological sample from said individual,

b) determining the mannose-binding lectin (MBL) genotype of said individual,

c) predicting the risk of said individual of developing sepsis by correlating the MBL genotype determined in step b) with a predefined risk value associated with said particular MBL genotype.

A predefined risk value associated with said particular MBL genotype in the present context can be divided in a high risk and a low risk for developing sepsis in said individual having SIRS.

Typically, a high risk relates to a MBL genotype that is characterised in having at least one variant structural allele of the MBL gene and/or having two low-expression regulatory alleles of the MBL gene in said samples.

All other MBL genotypes are predefined not to have a higher risk.

In the present context SIRS, sepsis, severe sepsis, and septic shock described defined as per the recommendations of the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference (Bone et al. 1992) and as described below.

In general Systemic Inflammatory Response Syndrome (SIRS) relates to an individual having at least two indications of systemic inflammation response syndrome (SIRS) selected from the group consisting of

(1) a core temperature of ≥ 38°C or ≤36°C;

(2) a heart rate of >90 beats/min;

(3) a respiratory rate of ≥ 20 breaths/min, a $PaCO_2$ ratio of ≤ 4.3 kPa (32 mm Hg), or a need for mechanical ventilation;

(4) a white blood cell count of ≥ $12.0x10^9$ cells/l or ≤$4.0x10^9$ cells/l or a differential count showing > 10% immature neutrophils

As the skilled addressee would recognise, any temperature above or equal to 38°C relates to temperatures from 38.0°C to any temperature by which the individual is capable of retain life, such as 38.1°C, 38.2°C, 38.3°C, 38.4°C, 38.5°C, 38.6°C, 38.7°C, 38.8°C, 38.9°C, 39.0°C, 39.1°C, 39.2°C, 39.3°C, 39.4°C, 39.5°C, 39.6°C, 39.7°C, 39.8°C, 39.9°C, 40.0°C, 40.1°C, 40.2°C, 40.3°C, 40.4°C, 40.5°C, 40.6°C, 40.7°C, 40.8°C, 40.9°C, 41.0°C, 41.1°C, 41.2°C, 41.3°C, 41.4°C, 41.5°C, 41.6°C, 41.7°C, 41.8°C, 41.9°C, or 42.0°C. Furthermore, any temperature below or equal to 36°C relates to temperatures from 36.0°C to any temperature by which the individual is capable of retain life, such as 35.9°C, 35.8°C, 35.7°C, 35.6°C, 35.5°C, 35.0°C, 34.5°C, 34.0°C, 33.5°C, 33.0°C, 32.5°C, 32.0°C, 31°C, 30°C, 29°C, 28°C, 27°C, 26°C, 25°C, 24°C, 23°C, 22°C, 21°C, 20°C, 21°C, 20°C, 19°C, 18°C, 17°C, 16°C, 15°C, 14°C, 13°C, 12°C, 11°C, or 10°C.

As the skilled addressee would recognise, a heart rate above 90 beats/min relates to a heart rate from 91 beats/min to 240 such as but not limited to a heart beat of 91 beats/min, 92 beats/min, 93 beats/min of 94 beats/min, 95 beats/min, 100 beats/min, of 105 beats/min, 110 beats/min, 120 beats/min, 125 beats/min, 130 beats/min, 135 beats/min, 140 beats/min of 145 beats/min, 150 beats/min, 155 beats/min, of 160 beats/min, 165 beats/min, 170 beats/min, 180 beats/min, 185 beats/min, 190 beats/min, 195 beats/min of 200 beats/min, 205 beats/min, 210 beats/min, 215 beats/min, or 220 beats/min.

As the skilled addressee would recognise, a respiratory rate of more than or equal to 20 breaths/min, relates to a respiratory rate of 20 breaths/min, 21 breaths/min, 25 breaths/min, 30 breaths/min, 35 breaths/min, 40 breaths/min, 50 breaths/min, 60 breaths/min, 70 breaths/min, 100 breaths/min, 120 breaths/min, 150 breaths/min, 175 breaths/min, 200 breaths/min, 250 breaths/min, 275 breaths/min, 300 breaths/min, 350 breaths/min, 375 breaths/min or 400 breath/min.

As the skilled addressee would recognise, a respiratory rate with a PaCO2 ratio of below or equal to 4.3 kPa (32 mm Hg), includes PaCO2 ratios of 4.3 kPa, 4.2 kPa, 4.1 kPa, 4.0 kPa, 3.9 kPa, 3.8 kPa, 3.7 kPa, 3.6 kPa, 3.5 kPa, 3.4 kPa, 3.3 kPa, 3.2 kPa, 3.1 kPa, 3.0 kPa, 2.9 kPa, 2.8 kPa, 2.7 kPa, 2.6 kPa, 2.5 kPa, 2.4 kPa, 2.3 kPa, 2.2 kPa, 2.1 kPa, 2.0 kPa, 1.9 kPa, 1.8 kPa, 1.7 kPa, 1.6 kPa, 1.5 kPa, 1.4 kPa, 1.3 kPa, 1.2 kPa, 1.1 kPa, 1.0 kPa, 0.9 kPa, 0.8 kPa, 0.7 kPa, 0.6 kPa, 0.5 kPa, 0.4 kPa, 0.3 kPa, 0.2 kPa, or 0.1 kPa.

As the skilled addressee would recognise the need for mechanical ventilation is an individual assessment by the individual trained medical personnel.

As the skilled addressee would recognise, a white blood cell count of above or equal to $12.0x10^9$ cells/l or less than or equal to $4.0x10^9$ cells/l relates to white blood cell counts of $12.0x10^9$ cells/l, $12.1x10^9$ cells/l, $12.2x10^9$ cells/l, $12.3x10^9$ cells/l, $12.4x10^9$ cells/l, $12.5x10^9$ cells/l, $13.0x10^9$ cells/l, $13.5x10^9$ cells/l, $14.0x10^9$ cells/l, $14.5x10^9$ cells/l, $15.0x10^9$ cells/l, $16.0x10^9$ cells/l, $17.0x10^9$ cells/l, $18.0x10^9$ cells/l or above, or $4.0x10^9$ cells/l, $3.9 x10^9$ cells/l, $3.8 x10^9$ cells/l, $3.7 x10^9$ cells/l, $3.6x10^9$ cells/l, $3.5 x10^9$ cells/l, $3.4 x10^9$ cells/l, $3.3 x10^9$ cells/l, $3.2x10^9$ cells/l, $3.1x10^9$ cells/l, $3.0x10^9$ cells/l, $2.5x10^9$ cells/l, $2.0x10^9$ cells/l, $1.5x10^9$ cells/l, $1.0x10^9$ cells/l, $0.5x10^9$ cells/l or $0.1x10^9$ cells/l.

In a particular preferred embodiment, the invention also relates a method according to the present invention, wherein said individual has a white blood cell count of above or equal to $10.0x10^9$ cells/l, since any value above the average normal of approximately $9.0x10^9$ cells/l could be indicative of a higher risk. Thus, a white blood cell count of $10.0x10^9$ cells/l, $10.1x10^9$ cells/l, $10.2x10^9$ cells/l, $10.3x10^9$ cells/l, $10.4x10^9$ cells/l, $10.5x10^9$ cells/l, $10.6x10^9$ cells/l, $10.7x10^9$ cells/l, $10.8x10^9$ cells/l, $10.9x10^9$ cells/l, $11.0x10^9$ cells/l, $11.1x10^9$ cells/l, $11.2x10^9$ cells/l, $11.3x10^9$ cells/l, $11.4x10^9$ cells/l, $11.5x10^9$ cells/l, $11.6x10^9$ cells/l, $11.7x10^9$ cells/l, $11.8x10^9$ cells/l, or $11.9x10^9$ cells/l is within of the scope of the present invention.

As the skilled addressee would recognise a differential count showing above 10% immature neutrophils relates to 10.5% immature neutrophils, 11% immature neutrophils, 12% immature neutrophils, 13% immature neutrophils, 14% immature neutrophils, 15% immature neutrophils, 20% immature neutrophils or above.

The sepsis criteria in the present context is SIRS with a documented infection or a clinically suspected infection as in postoperative intra-abdominal sepsis in which the intestinal tract had either been perforated or required partial resection for ischemia, or intestinal content appeared intra-abdominally due to leak from prior gut anastomosis; or radiographic evidence of pneumonia in association with purulent sputum.

Infection is considered documented by a positive culture or convincing gram stain no more than 3 days prior to admittance to intensive care.

Severe sepsis is defined as sepsis and either hypotension or evidence of hypoperfusion and organ dysfunction developing within 24 hour of study enrolment. Individual would have to meet at least one of the following criteria to be defined as having organ dysfunction;

1) arterial systolic blood pressure <90 mm Hg for at least one hour despite appropriate fluid resuscitation, or vasopressor therapy to maintain a systolic blood pressure >90 mm Hg;

2) urine output <0.5 ml/kg for >1 hour despite hydration;

3) PaO_2 / FiO_2 ratio ≤40 kPa (300 mmHg);

4) an acute alteration in mental status (GCS <14);

5) unexplained metabolic acidosis with pH ≤7.30 or base deficit ≥ 5.0 mmol/l in association with an increased plasma lactate level ≥1.6 mmol/l;

6) hepatobiliary dysfunction with serum bilirubin >34 µmol/l and no evidence of pre-existing hepatobiliary disease.

Septic shock was defined as sepsis with hypotension in combination with one of the other criteria of acute organ dysfunction. Patients were excluded from participation if any of the following conditions were present: neutrophil count of less than 1.0×10^9/l prior to the onset of sepsis; infections associated with burns; documented or suspected recent acute myocardial infarction; or lack of commitment to full life-support measures by the primary physician.

Multiple organ failure (MOF) is a common cause of death in surgical critical care units. MOF means the presence of altered organ function in an ill patient such that homoeostasis cannot be maintained without intervention. Initially it was believed that progressive organ failure was related to uncontrolled infection. Recently it is clear that the noninfectious insults such as trauma, pancreatitis, burns and massive transfusion may produce a

syndrome of multiple organ dysfunction clinically indistinguishable from infectious cause. An awareness of the setting of the various problems contributing to the syndrome allows one to predict the possibilities of organ failure and to maintain support to these organs before they fail. Sepsis and systemic inflammatory response syndrome-induced severe disruption of microcirculation and consecutive tissue hypoxia is considered a key factor in the development of organ dysfunction and multiple organ failure. Thus, the present invention may also predict the risk of an individual having SIRS will develop multiple organ failure or multiple organ dysfunction.

MBL genotype

The method of the present invention determines the presence of both variant and normal polymorphism of the MBL gene in said sample.

MBL single nucleotide polymorphism's (SNPs) in form of the structural variants named B (codon 54), C (codon 57), and D (codon 52) as well as the regulatory variants named H/L (-550), X/Y (-221), and P/Q (+4) were in the present invention typed by PCR using sequence specific priming (PCR-SSP). As internal positive control in the presently preferred embodiment, the present inventors included a PCR covering exon 4 of the mbl2 gene as shown in the examples below.

Figure 1 shows seven patterns necessary for covering all combinations of each of the six complementary reactions. The typing system was validated by automated sequencing (ABI 3100 platform) of PCR products from the seven control samples covering all of the polymorphic positions and in addition, by comparing the typing system with the previously used typing techniques.

Although the typing was performed as SNP-typing the results were combined in haplotypes, based on strong linkage disequilibrium between the SNPs that gives the seven known haplotypes:

> Four functional haplotypes *LXPA*, *LYPA*, *LYQA*, and *HYPA* (the normal allele is designated "*A*"), and three defective haplotypes; *LYPB*, *LYQC*, and *HYPD*.

All three structural variant alleles (*B*, *C*, and *D*) have a considerable effect on MBL concentrations and to avoid small groups, the three alleles were grouped in one category called allele "*O*" for statistical analyses.

Likewise, for statistical analyses described below, the present inventors only included the *X/Y* promoter variation at position -221.

The *X* variant is always found on a functional haplotype (*LXPA*) and has been shown to have a down regulating effect on MBL expression. Thus, the following six MBL genotypes/haplotypes is defined:

the *A/A* group: two normal structural alleles with high-expression promoter activity in position -221 (*YA/YA*) or one high-expression promoter and one low-expression promoter (*YA/XA*) or two low-expression promoters (*XA/XA*);

the *A/0* group: one variant structural allele (i.e. defective allele) and one normal structural allele regulated by a high-expression promoter (*YA/O*) or a low-expression promoter (*XA/O),* and the

O/O group with two defective structural alleles.

In one embodiment, the present invention relates to a method according to the present invention, wherein the MBL genotype has at least one variant structural allele of the MBL gene.

In one embodiment, the present invention relates to a method according to the present invention, wherein the MBL has two low-expression regulatory alleles of the MBL gene.

In some embodiments of the invention the regulatory allele may be situated in the promoter region of the MBL gene.

In a presently preferred embodiment, the present invention relates to a method according the present invention, wherein the polymorphism lies in position 602, which generates a low-expression regulatory allele when position 602 has a base C substitution (SEQ ID NO: 26 (GeneBank, ID No. Y16580), or generates a high-expression regulatory allele when position 602 has a base G substitution (SEQ ID NO: 24 (GeneBank, ID No. Y16581).

In another presently preferred embodiment, the present invention relates to a method according to the present invention, wherein the polymorphism lies in codon 52 in position 1045, which generates a variant structural allele, when position 1045 has a base T (SEQ ID NO: 25 (GeneBank, ID No. Y16582)) or generates a normal structural allele, when position 1045 has a base C (SEQ ID NO: 24 (GeneBank, ID No. Y16581)).

In another presently preferred embodiment, the present invention relates to a method according to the present invention, wherein the polymorphism lies in codon 54 in position 1052, which generates a variant structural allele, when position 1052 has a base A (SEQ ID NO: 23 (GeneBank, ID No. Y16579)) or generates a normal structural allele, when position 1052 has a base G (SEQ ID NO: 24 (GeneBank, ID No. Y16581)).

In another presently preferred embodiment, the present invention relates to a method according to the present invention, wherein the polymorphism lies in codon 57 in position 1055, which generates a variant structural allele, when position 1055 has a base A (SEQ ID NO: 22 (GeneBank, ID No. Y16576)) or generates a normal structural allele, when position 1055 has a base G (SEQ ID NO: 20 (GeneBank, ID No. Y16576)).

It should be understood that any feature and/or term discussed above in connection with "wild-type" according to the invention apply by analogy to "normal" according to the invention.

The allele may be normal (see for example SEQ ID NO: 20, 21, 22 and 26), or it may be a variant, such as a structural variant or a non-structural variant.

By structural allele is meant a polymorphic position situated in the protein coding part of the gene.

By variant structural allele is meant a structural allele that differs from the wild-type structural allele by a substitution and/or a deletion and/or an insertion of a nucleotide or more nucleotides.

A wild type gene relates to a gene a functionally normal protein, whereas a variant gene encodes a variant protein with a function and/or structure that differs from that of a wild type protein.

By "structural variant" is meant a polymorphism in the part of the MBL gene responsible for encoding the protein.

By "non-structural" variant is meant an allele not associated with part of the MBL gene responsible for encoding the protein, such as a regulatory allele.

The term "regulatory variant" means a polymorphism in a part of the MBL gene associated with regulating the transcription or translation of the gene.

In the present context "MBL" also covers analogues of MBL. An analogue is a compound (or molecule) that is a (chemical) structural derivative of MBL. It is also used to describe a molecule which may be structurally similar (but not identical) to another, and which exhibits many or some of the same biological functions of MBL. An analogue is to be understood as being any peptide sequence capable of the same biological functions as wild-type MBL, including recombinant MBL.

In one embodiment the MBL genotype of an individual is determined according to at least two alleles by determining the presence of at least one structural allele of the MBL gene and/or the presence of at least one non-structural allele of the MBL gene of said individual.

In yet a further embodiment the MBL gene is MBL2 (see SEQ ID NO: 20, 21, 22, 26).

Non-structural variant

According to the invention in one aspect the non-structural variant is in the promoter region of the MBL gene.

In another aspect the non-structural variant is in the regulatory region of the MBL gene.

The non-structural allele may be selected among the following sequences:

In one embodiment the non-structural allele has a base C substitution in position 602 of SEQ ID NO: 26 (GeneBank, ID No. Y16580) or a base G substitution in position 602 of SEQ ID NO: 21 (GeneBank, ID No. Y16577).

In another embodiment the non-structural allele has a base C substitution in position 273 of SEQ ID NO: 21 (GeneBank, ID No. Y16577) or a base G substitution in position 273 of SEQ ID NO: 25 (GeneBank, ID No. Y16581).

In yet another embodiment the non-structural allele has a base A substitution in position 396 of SEQ ID NO: 21 (GeneBank, ID No. Y16577) or a base C substitution in position 396 of SEQ ID NO: 20 (GeneBank, ID No. Y16576).

Further, in another embodiment the non-structural allele has a base A substitution in position 474 of SEQ ID NO: 21 (GeneBank, ID No. Y16577) or a base G substitution in position 474 of SEQ ID NO: 20 (GeneBank, ID No. Y16576).

In yet a further embodiment the non-structural allele has a base A substitution in position 487 of SEQ ID NO: 21 (GeneBank, ID No. Y16577) or a base G substitution in position 487 of SEQ ID NO: 20 (GeneBank, Seq ID No. Y16576).

In another embodiment the non-structural allele has a deletion in base sequence AAAGAG in position 495-500 of SEQ ID NO: 21 (GeneBank, ID No. Y16577).

In yet another embodiment the non-structural allele has a base C substitution in position 753 of SEQ ID NO: 21 (GeneBank, ID No. Y16577) or a base T substitution in position 747 of SEQ ID NO: 20 (GeneBank, Seq ID No. Y16576).

In another embodiment the non-structural allele has a base C substitution in position 826 of SEQ ID NO: 21 (GeneBank, ID No. Y16577) or a base T substitution in position 820 of SEQ ID NO: 20 (GeneBank, ID No. Y16576).

Structural allele

Another aspect of the invention concerns at least one structural allele. In one embodiment the at least one structural allele has a substitution in codon 54 in base G in position 1052 of SEQ ID NO: 21 (GeneBank, ID No. Y16577) or in base A in position 1052 of SEQ ID NO: 23 (GeneBank, ID No. Y16579).

In another embodiment the at least one structural allele has a substitution in codon 57 in base G in position 1055 of SEQ ID NO: 20 (GeneBank, ID No. Y16576) or in base A in position 1055 of SEQ ID NO: 22 (GeneBank, ID No. Y16578).

In yet another embodiment the at least one structural allele has a substitution in codon 52 in base C in position 1045 of SEQ ID NO: 24 (GeneBank, ID No. Y16581) or in base T in position 1045 of SEQ ID NO: 25 (GeneBank, ID No. Y16582).

High/low expression promoters

According to the invention the variety of the genotypes may be used in the determination of the risk factor of an individual of developing sepsis, severe sepsis, or septic shock.

According to the invention the determination of the risk factor of an individual of developing sepsis, severe sepsis, or septic shock may be applied to both healthy and sick individuals. It is envisioned that the above risk factor of an individual may be registered in a file, for example in a hospital for use in situations where an individual has been injured.

The risk information would provide valuable information, which may be used in determining a treatment strategy of said individual.

In one embodiment of the invention the genotype comprises two normal structural alleles regulated by two high-expression promoters.

In another embodiment the genotype comprises two normal structural alleles regulated by two low-expression promoters.

By the term "high-expression promoters" is meant promoter alleles associated with a high MBL protein expression/concentration.

In the present context the term "low-expression promoters" is meant promoter alleles associated with a low MBL protein expression/concentration.

In yet a further embodiment of the invention the genotype comprises two normal structural alleles regulated by one low-expression promoter and one high-expression promoter.

Furthermore, in another embodiment the genotype comprises one normal structural allele regulated by one high-expression promoter and one variant structural allele.

In yet another embodiment the genotype comprises one normal structural allele regulated by one low-expression promoter and one variant structural allele.

In another embodiment the genotype comprises two variant structural alleles.

Risk value

The nucleic acid sequences and/or the MBL concentration according to the present invention makes it possible to estimate sepsis risk in an individual. In the former embodiment the risk estimation is based on sequence polymorphism originating from specific regions of the MBL gene. In the latter case the MBL concentration may be determined using conventional methods mentioned further below.

By sequence polymorphism is understood any single nucleotide, tandem repeat, insert, deletion or block polymorphism, which varies among humans, whether it is of biological importance or not.

The estimation of the risk of sepsis has a number of important applications:

Companies, hospitals or other institutions can with the methods of the present invention offer a service to help individuals determine whether they belong to a risk group and if so provide treatment accordingly. One possible application could be in an emergency situation, such as in an ambulance after an accident where emergency staff may determine the risk factor of the injured individual and provide a treatment accordingly.

Alternatively, individuals with reasons to suspect that they may be at risk of developing sepsis would be able to clarify their situation and if possible take protective action.

The present invention concerns a method of determining the risk factor of a person of developing sepsis, severe sepsis or septic shock. In the present context such risk factor is expressed as an arbitrary risk value, which has been defined prior to determining the actual risk (likelihood) of an individual developing sepsis, severe sepsis, or septic shock.

One embodiment relates to various risk values associated with particular MBL genotypes.

In one embodiment of the invention the particular MBL genotype associated with a low risk value comprises two normal MBL structural alleles regulated by two high-expression promoters.

In another embodiment the particular MBL genotype associated with a medium-low risk value comprises two normal MBL structural alleles regulated by one low-expression promoter and one high-expression promoter.

In yet another embodiment the particular MBL genotype associated with a medium risk value comprises two normal MBL structural alleles regulated by two low-expression promoters.

In a further embodiment of the invention the particular MBL genotype associated with a medium-high risk value comprises one normal structural MBL allele regulated by a high-expression promoter and one variant structural allele.

In yet a further embodiment the particular MBL genotype associated with a high-risk value comprises one normal structural allele regulated by a low-expression promoter and one variant structural allele.

In one aspect of the invention the particular MBL genotype associated with an ultra-high risk value comprises two MBL variant structural alleles.

Amplification

It will be apparent to the person skilled in the art that there are a large number of analytical procedures, which may be used to detect the presence or absence of the structural and/or non-structural variant nucleotides mentioned herein. Nucleic acid from any nucleated cell can be used as the starting point for such assay techniques and may be isolated according to standard nucleic acid preparation procedures well known to those of skill in the art. In general the detection of allelic variation requires an amplification reaction and, a signal generation system and a mutation discrimination technique. Table A lists a number of mutation detection techniques, some are based on the PCR technique. These may be used in combination with a number of signal generation systems a selection of which is listed in Table B. Further amplification techniques are listed in Table C. Many current methods for the detection of allelic variation are reviewed by Nollau et al., Clin. Chem. 43, 1114-1120, 1997; and in standard textbooks, for example "Laboratory Protocols for Mutation Detection", Ed. by U. Landegren, Oxford University Press, 1996 and "PCR", 2.sup.nd Edition by Newton & Graham, BIOS Scientific Publishers Limited, 1997.

The variant alleles of the present invention may be subjected to amplification, thus a presently preferred embodiment relates to a method according to the present invention, wherein the at least one structural allele or the at least one regulatory allele is subjected to amplification.

The amplification may be performed by an amplification method selected from the group consisting of polymerase chain reaction (PCR), Ligase Chain Reaction (LCR), Nucleic Acid Sequence-Based Amplification (NASBA), strand displacement amplification, rolling circle amplification, and T7-polymerase amplification.

In a preferred embodiment the amplification is performed by PCR.

In a presently preferred embodiment the amplification is carried out by means of sequence-specific primers (SSP).

Table A

Abbreviations:

ALEX .™.	Amplification refractory mutation system linear extension
APEX	Arrayed primer extension
PCR-ARMS .™	Amplification refractory mutation system

b-DNA	Branched DNA
CMC	Chemical mismatch cleavage
bp	base pair
PCR-COPS	Polychainreaction-competitive oligonucleotide priming system
DGGE	Denaturing gradient gel electrophoresis
FRET	Fluorescence resonance energy transfer
LCR	Ligase chain reaction
PCR-MASDA	Polychain reaction-multiple allele specific diagnostic assay
PCR-NASBA	Polychain reaction-nucleic acid sequence based amplification
OLA	Oligonucleotide ligation assay
PCR	Polymerase chain reaction
PTT	Protein truncation test
RFLP	Restriction fragment length polymorphism
PCR-SDA	Polychain reaction-strand displacement amplification
SNP	Single nucleotide polymorphism
SSCP	Single-strand conformation polymorphism analysis
SSP	Sequence Specific Priming
SSR	Self sustained replication
TGGE	Temperature gradient gel electrophoresis

Table B illustrates various mutation detection techniques capable of being used for SNP detection.

Table B

General techniques: DNA sequencing, Sequencing by hybridisation, SNAPshot.

Scanning techniques: SSCP, DOGE, TGGE, Cleavase, Heteroduplex analysis, CMC, Enzymatic mismatch cleavage, DGGE.

Hybridisation Based techniques: Solid phase hybridisation: Dot blot hybridization, MASDA, Reverse dot blot hybridization, Oligonucleotide arrays (DNA Chips)

Solution phase hybridisation: Taqman.TM.--U.S. Pat. No. 5,210,015 & 5,487,972 (Hoffmann-La Roche), Molecular Beacons--Tyagi et al (1996), Nature Biotechnology, 14, 303; WO 95/13399 (Public Health Inst., New York), Lightcycler, optionally in combination with FRET.

Extension Based: ARMS.™., PCR-SSP, ALEX.™.--European Patent No. EP 332435 B1 (Zeneca Limited), COPS--Gibbs et al (1989), Nucleic Acids Research, 17, 2347.

Incorporation Based: Mini-sequencing, APEX

Restriction Enzyme Based: RFLP, Restriction site generating PCR

Ligation Based: OLA

Other: Invader assay

Various Signal Generation or Detection Systems are listed below:
Fluorescence: FRET, Fluorescence quenching, Fluorescence polarisation--United Kingdom Patent No. 2228998 (Zeneca Limited)

Other: Chemiluminescence, Electrochemiluminescence, Raman, Radioactivity, Colorimetric, Hybridisation protection assay, Mass spectrometry
Below are examples of further amplification techniques.

SSR, NASBA, LCR, SDA, b-DNA

Preferred mutation detection techniques include ARMS.™. or PCR-SSP, ALEX.™., COPS, Taqman, Molecular Beacons, RFLP, and restriction site based PCR and FRET techniques.

Particularly preferred methods include FRET, Taqman, ARMS.™. or PCR-SSP and RFLP based methods.

In the method of the invention the presence of the at least one variant allele is determined by hybridising a primer to a target nucleic acid sequence comprising at least one structural allele or at least one non-structural allele or at least one regulatory allele, or hybridising to any complementary sequence of said alleles.

In one embodiment of the invention the amplification is carried out by means of sequence-specific primers (SSP).

Time
The possible fatal outcome of sepsis, severe sepsis and septic shock in an individual having SIRS necessitate a fast and reliable overview of said individuals genetype in order to select

and initiate the proper treatment in due time, thus it is an object of preferred embodiments of the present invention to provide the results as quickly as possible.

In a presently preferred embodiment the invention relates to a method according to present invention, wherein said method is carried out within 168 hours of the initiation of the indications of SIRS, such as but not limited to 150 hours of the initiation of the indications of SIRS, 140 hours of the initiation of the indications of SIRS, 130 hours of the initiation of the indications of SIRS, 120 hours of the initiation of the indications of SIRS, 110 hours of the initiation of the indications of SIRS, 100 hours of the initiation of the indications of SIRS, 90 hours of the initiation of the indications of SIRS, 80 hours of the initiation of the indications of SIRS, 70 hours of the initiation of the indications of SIRS, 60 hours of the initiation of the indications of SIRS, 50 hours of the initiation of the indications of SIRS,48 hours of the initiation of the indications of SIRS, 36 hours of the initiation of the indications of SIRS, 24 hours of the initiation of the indications of SIRS, 20 hours of the initiation of the indications of SIRS, 18 hours of the initiation of the indications of SIRS, 16 hours of the initiation of the indications of SIRS, 12 hours of the initiation of the indications of SIRS, 10 hours of the initiation of the indications of SIRS, 6 hours of the initiation of the indications of SIRS, 2 hours of the initiation of the indications of SIRS or less.

According to the invention the typing of the polymorphism is performed within at the most 5 hours, such as within at the most 4.5 hours, within at the most 4 hours, within at the most 3.5 hours, within at the most 3 hours, within at the most 2.5 hours, within at the most 2 hours, within at the most 1.5 hours, within at the most 60 minutes, within at the most 50 minutes, for example within at the most 40 minutes, such as within at the most 30 minutes, for example within at the most 20 minutes, such as at the most 10 minutes.

In one embodiment of the invention the PCR-SSP technique is used. In another embodiment the mutation detection technique used is without an amplification step.

In a preferred embodiment, mutations or polymorphism's can be detected by using a microassay of nucleic acid sequences immobilized to a substrate or "gene chip" (see, e.g. Cronin, et al., 1996, Human Mutation 7:244-255).

Additionally, it is possible to perform such allele expression assays "in situ", i.e. directly on tissue sections (fixed and/or frozen) of tissue obtained from biopsies or resections of individuals, such that no nucleic acid purification is necessary. Nucleic acid reagents such as those described above may be used as probes and/or primers for such in situ

procedures (see, for example, Nuovo, G. J., 1992, "PCR In Situ Hybridization: Protocols And Applications", Raven Press, NY).

Mutations outside the region

The present invention may combine the result of MBL2 gene polymorphism within the codons 52, 54 and 57 with gene polymorphism's outside these codons in order to increase the probability of the significance of the correlation between MBL polymorphism's and sepsis risk.

Primers

The risk assessment of the invention may be conducted by means of at least one nucleic acid primer or probe, such as a primer or probe of DNA, RNA or a nucleic acid analogue such as peptide nucleic acid (PNA) or locked nucleic acid (LNA). The nucleotide primer or probe is preferably capable of hybridising to a subsequence of the region corresponding to any of the MBL haplotype sequences, or a part thereof, or a complementary region thereof.

The design of such probes will be apparent to the molecular biologist of ordinary skill. Such probes are of any convenient length such as up to 50 bases, up to 40 bases, more conveniently up to 30 bases in length, such as for example 8-25 bases in length. In general such probes will comprise base sequences entirely complementary to the corresponding wild type or variant locus in the MBL gene. However, if required one or more mismatches may be introduced, provided that the discriminatory power of the oligonucleotide probe is not unduly affected. The probes of the invention may carry one or more labels to facilitate detection.

In one embodiment of the invention the amplification employs the following sequence-specific primers:

5' primer: AGTCGACCCAGATTGTAGGACAGAG (SEQ ID NO:1)

3' primer: CCTTTTCTCCCTTGGTGC (SEQ ID NO:2)

In another embodiment of the invention the amplification employs the following sequence-specific primers:

5' primer: GCAAAGATGGGCGTGATGA (SEQ ID NO:3)

3' primer: GGGCTGGCAAGACAACTATTA (SEQ ID NO:4)

In a further embodiment of the invention the amplification employs the following sequence-specific primers:

5' primer: AGTCGACCCAGATTGTAGGACAGAG (SEQ ID NO:1)
3' primer: CCTGGTTCCCCCTTTTCTC (SEQ ID NO:5)

In another aspect the amplification employs the following sequence-specific primers:
5' primer: AGTCGACCCAGATTGTAGGACAGAG (SEQ ID NO:1)
3' primer: ACCTGGTTCCCCCTTTTCTT (SEQ ID NO:6)

In yet another aspect the amplification employs the following sequence-specific primers:
5' primer: AGTCGACCCAGATTGTAGGACAGAG (SEQ ID NO:1)
3' primer: TCCCTTGGTGCCATCACG (SEQ ID NO:7)

The invention concerns in one aspect the amplification employing the following sequence-specific primers:
5' primer: AGTCGACCCAGATTGTAGGACAGAG (SEQ ID NO:1)
3' primer:CTCCCTTGGTGCCATCACA (SEQ ID NO:8)

In another aspect the invention discloses the amplification employing the following sequence-specific primers:
5' primer: CATTTGTTCTCACTGCCACC(SEQ ID NO:9)
3' primer: CTCAGGGAAGGTTAATCTCAG (SEQ ID NO:10)

Further, in yet another embodiment the amplification employs the following sequence-specific primers:
5' primer: CATTTGTTCTCACTGCCACG (SEQ ID NO:11)
3' primer: CTCAGGGAAGGTTAATCTCAG (SEQ ID NO:10)

In one aspect the amplification employs the following sequence-specific primers:
5' primer: GGCTTAGACCTATGGGGCTA (SEQ ID NO:12)
3' primer: GCTTCCCCTTGGTGTTTTAC (SEQ ID NO:13)

In another aspect the amplification employs the following sequence-specific primers:
5' primer: GGCTTAGACCTATGGGGCTA (SEQ ID NO:12)
3' primer: GCTTCCCCTTGGTGTTTTAG (SEQ ID NO:14)

In yet another aspect the amplification employs the following sequence-specific primers:
5' primer: TAGGACAGAGGGCATGCTC(SEQ ID NO:15)
3' primer: AGGATCCAGGCAGTTTCCTCTGGAAGG (SEQ ID NO:16)

In yet a further aspect the amplification employs the following sequence-specific primers:

5' primer: TAGGACAGAGGGCATGCTT (SEQ ID NO:17)
3' primer: AGGATCCAGGCAGTTTCCTCTGGAAGG (SEQ ID NO:16)

In another aspect the amplification employs the following sequence-specific primers:

5' primer: GAGTTTCACCCACTTTTTCACA (SEQ ID NO:18)
3' primer: GCCTGAGTGATATGACCCTTC (SEQ ID NO:19)

SEQ ID NO:18 and SEQ ID NO:19 are control primers.

A presently preferred embodiment of the present invention relates to a method according to the present invention, wherein the amplification employs the following sequence-specific primer pairs selected form the group consisting of at least one of:

5' primer: AGTCGACCCAGATTGTAGGACAGAG (SEQ ID NO:1)
3' primer: CCTTTTCTCCCTTGGTGC (SEQ ID NO:2),

5' primer: GCAAAGATGGGCGTGATGA (SEQ ID NO:3)
3' primer: GGGCTGGCAAGACAACTATTA (SEQ ID NO:4),

5' primer: AGTCGACCCAGATTGTAGGACAGAG (SEQ ID NO:1)
3' primer: CCTGGTTCCCCCTTTTCTC (SEQ ID NO:5),

5' primer: AGTCGACCCAGATTGTAGGACAGAG (SEQ ID NO:1)
3' primer: ACCTGGTTCCCCCTTTTCTT (SEQ ID NO:6),

5' primer: AGTCGACCCAGATTGTAGGACAGAG (SEQ ID NO:1)
3' primer: TCCCTTGGTGCCATCACG (SEQ ID NO:7),

5' primer: AGTCGACCCAGATTGTAGGACAGAG (SEQ ID NO:1)
3' primer:CTCCCTTGGTGCCATCACA (SEQ ID NO:8),

5' primer: CATTTGTTCTCACTGCCACC(SEQ ID NO:9)
3' primer: CTCAGGGAAGGTTAATCTCAG (SEQ ID NO:10),

5' primer: CATTTGTTCTCACTGCCACG (SEQ ID NO:11)
3' primer: CTCAGGGAAGGTTAATCTCAG (SEQ ID NO:10),

5' primer: GAGTTTCACCCACTTTTTCACA (SEQ ID NO:18)
3' primer: GCCTGAGTGATATGACCCTTC (SEQ ID NO:19).

Measuring the MBL concentration

One aspect of the invention concerns a method of predicting whether an individual will develop sepsis, severe sepsis, or septic shock comprising determining the concentration of MBL in a biological sample from an individual, such as a blood sample, for example a serum sample. The determination may in one aspect be performed directly in the blood stream of said individual. In a further aspect the MBL concentration is determined in a blood sample collected from said individual.

In another embodiment the invention relates to a method of predicting whether an individual having SIRS will develop sepsis comprising the steps of:

a) providing a biological sample from said individual,

b) determining the MBL concentration in said individual,

c) predicting the risk of said individual of developing sepsis by correlating the MBL concentration of step b) with a predefined risk value associated with said particular MBL concentration.

In the present context any MBL concentration below 1.5 mg/l relates to a higher risk for said individual having SIRS developing sepsis, such as but not limited to below 1.45 mg/l, below 1.40 mg/l, below 1.35 mg/l, below 1.30 mg/l, below 1.25 mg/l, below 1.20 mg/l, below 1.15 mg/l, below 1.10 mg/l, below 1.05 mg/l, below 1.00 mg/l, below 0.95 mg/l, below 0.90 mg/l, below 0.85 mg/l, below 0.80 mg/l, below 0.75 mg/l, below 0.70 mg/l, below 0.65 mg/l, below 0.60 mg/l, below 0.50 mg/l, below 0.40 mg/l, below 0.35 mg/l, below 0.30 mg/l, below 0.25 mg/l, below 0.20 mg/l, below 0.15 mg/l, 0.10 mg/l or no detectable MBL concentration.

In another embodiment the invention relates to a method of predicting whether an individual will develop sepsis, severe sepsis, or septic shock comprising the steps of:

a) providing a biological sample from said individual,

b) determining the mannose-binding lectin (MBL) genotype of said individual, and determining the concentration of MBL in said individual,

c) predicting the risk of said individual of developing sepsis, severe sepsis or septic shock by correlating the MBL genotype or the MBL concentration of step b) with a

predefined risk value associated with said particular MBL genotype or said particular MBL concentration.

Particular MBL genotype of an individual will be reflected in the level of MBL protein concentration in for example blood.

In one embodiment the MBL concentration is determined by the means of for example, but not exclusively, ELISA, RIA or TRIFMA.

In another embodiment the MBL concentration is determined by determining the activity of MASP (MBL associated serine proteases).

In yet another embodiment the biological sample is provided from an individual having at least one indication of systemic inflammation response syndrome (SIRS).

In another embodiment the biological sample is provided from an individual having at least one indication of systemic inflammation response syndrome (SIRS), sepsis or severe sepsis. The definition of these conditions can be found in the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference (Bone et al. 1992). The following are SIRS criteria: a core temperature of ≥ 38°C or ≤36°C; a heart rate of >90 beats/min; a respiratory rate of ≥20 breaths/min or a $PaCO_2$ of ≤4.3 kPa (32 mm Hg) or a need of mechanical ventilation; a white blood cell count of ≥12.0 10^9/l or ≤4.0 10^9/l or a differential count showing >10 percent immature neutrophils. Sepsis criteria are SIRS with a documented infection or a clinically suspected infection as in postoperative intra-abdominal sepsis in which the intestinal tract is either perforated or required partial resection for ischemia, or intestinal content appeared intra-abdominally due to leak from prior gut anastomosis; or radiographic evidence of pneumonia in association with purulent sputum. Infection is considered documented by a positive culture or convincing gram stain no more than 3 days prior to admittance to intensive care. Severe sepsis is defined as sepsis and either hypotension or evidence of hypoperfusion and organ dysfunction developing within 24 hour of study enrolment. Individuals must meet at least one of the following criteria to be defined as having organ dysfunction; 1) arterial systolic blood pressure <90 mm Hg for at least one hour despite appropriate fluid resuscitation, or vasopressor therapy to maintain a systolic blood pressure >90 mm Hg; 2) urine output <0.5 ml/kg for >1 hour despite hydration; 3) PaO_2 / FiO_2 ratio ≤40 kPa (300 mmHg); 4) an acute alteration in mental status (GCS <14); 5) unexplained metabolic acidosis with pH ≤7.30 or base deficit ≥ 5.0 mmol/l in association with an increased plasma lactate level ≥1.6 mmol/l; 6) hepatobiliary dysfunction with serum bilirubin >34 µmol/l with elevation of alkaline phosphatase, or serum transaminases beyond twice the upper limit of normal

and no evidence of pre-existing hepatobiliary disease. Septic shock is defined as sepsis with hypotension in combination with one of the other criteria of acute organ dysfunction.

The present inventors have found that individuals with SIRS have a high risk of developing sepsis provided that they carry variant alleles in the *mbl2* gene, which decrease the level of MBL antigen and/or functional MBL in the blood. Without being bound by theory MBL seems to be crucial in controlling systemic dissemination of different infectious agents in individuals with acute medical and surgical stress. This observation is also in agreement with the inventor's finding that an increased proportion of individuals carrying MBL variant alleles have a positive bacterial blood culture.

The finding that the frequency of MBL variant alleles is proportional to the severity of sepsis (severe sepsis and septic shock) indicates that persons lacking the buffering capacity of MBL towards initial microbial replication, not only is associated with susceptibility to infection but may also allow activation of host mechanisms central to the pathophysiology of the sepsis syndrome. In agreement with this view are several *in vitro* findings indicating that MBL may suppress the release of proinflammatory cytokines. Thus, MBL may have both a direct antimicrobial role and may also have a modulating effect on the inflammatory response. Recently, MBL has been shown to function as scavenger molecule towards cells undergoing apoptosis and necrosis.

During a hospital stay there is an increased risk of a fatal outcome of individuals carrying MBL variant alleles. The present invention provides a method for genetically determining the differences in the *mbl2* gene and thereby develop a prognostic method useful in for example hospitals. A rapid determination of a patient's MBL genotype is important in identifying individuals at risk of developing sepsis, severe sepsis or septic shock.

Moreover, since MBL substitution therapy now is possible is within the scope of the present invention to use MBL in prophylaxis and treatment of the sepsis syndrome.

One aspect of the invention relates to a method of predicting whether an individual having SIRS will develop sepsis comprising combining the two methods described in the present application.

Biological sample

In one embodiment of the invention the biological sample is body fluid, such as blood salvia, urine, faeces, cerebrospinal fluid, plasma and/or serum, for example the biological sample used in the present invention may be any suitable biological sample capable of providing the genetic material for use in the method.

In a presently preferred embodiment, the body fluid is blood.

In another embodiment the biological sample is a tissue sample, a sample of secretion, semen, ovum, a washing of a body surface (e.g. a buccal swap), a clipping of a body surface (hairs, or nails), such as wherein the cell is selected from white blood cells and tumour tissue.

It will be appreciated that the biological test sample may equally well be a nucleic acid sequence corresponding to the sequence in the test sample, that is to say that all or a part of the region in the sample nucleic acid may firstly be amplified using any convenient technique e.g. PCR before use in the analysis of MBL variation.

However, in a preferred embodiment of the invention the MBL variation analysis and the PCR are performed simultaneously, for example by using the PCR-SSP (ARMS.™ technique).

Treatment profile

According to the invention the present method comprises an additional step d) of assessing the risk of said individual of developing sepsis, severe sepsis or septic shock and accordingly determining a treatment profile for said individual.

The kit

A presently preferred embodiment of the present invention relates to a kit for predicting whether an individual will develop sepsis comprising:

at least one 5' primer selected from the group comprising the nucleic acid sequences as defined in SEQ ID NO.:1, SEQ ID NO.:3, SEQ ID NO.:9, SEQ ID NO.:11, and SEQ ID NO.:18.

at least one 3' primer selected from the group comprising the nucleic acid sequences as defined in SEQ ID NO.:2, SEQ ID NO.:4, SEQ ID NO.:5, SEQ ID NO.:6, SEQ ID NO.:7, SEQ ID NO.:8, SEQ ID NO.:10 and SEQ ID NO.:19 for amplification of variant alleles of the MBL gene.

According to another aspect of the present invention a prognostic kit comprising at least one prognostic primer of the invention and/or at least one allele-specific oligonucleotide primer or probe of the invention is provided.

The prognostic kit may comprise appropriate packaging and instructions for use in the methods of the invention. Such kit may further comprise appropriate buffer(s) and polymerase(s) such as thermostable polymerases, for example Taq DNA polymerase.

A preferred kit may comprise means for amplifying the relevant sequence such as primers, polymerase, deoxynucleotides, buffer, metal ions; and/or means for discriminating the polymorphism, such as one or a set of primers or probes hybridising to the polymorphic site, a sequence reaction covering the polymorphic site, an enzyme or an antibody; and/or a secondary amplification system, such as enzyme-conjugated antibodies, or fluorescent antibodies. The kit-of-parts preferably also comprises a detection system, such as a fluorometer, a film, an enzyme reagent or another highly sensitive detection device.

An antibody-based kit may comprise, for example: (1) a first antibody (e.g., attached to a solid support) which binds to a polypeptide of the invention; and optionally (2) a second different antibody which binds to either the polypeptide or to the first antibody and is conjugated to a detectable agent.

Another aspect of the invention concerns a kit for predicting whether an individual will develop sepsis, severe sepsis or septic shock. The kit comprises:

at least one 5' primer selected from the group comprising the nucleic acid sequences as defined in SEQ ID NO.:1, SEQ ID NO.:3, SEQ ID NO.:9, SEQ ID NO.:11, SEQ ID NO.:12, SEQ ID NO.:15, SEQ ID NO.:17, and SEQ ID NO.:18.

at least one 3' primer selected from the group comprising the nucleic acid sequences as defined in SEQ ID NO.:2, SEQ ID NO.:4, SEQ ID NO.:5, SEQ ID NO.:6, SEQ ID NO.:7, SEQ ID NO.:8, SEQ ID NO.:10, SEQ ID NO.:13, SEQ ID NO.:14, SEQ ID NO.:16, and SEQ ID NO.:19 for amplification of variant alleles of the MBL gene.

Label

In one embodiment of the invention the at least one primer of the kit comprises a detectable label.

The detectable may be selected from TEX, TET, TAM, ROX, R6G, ORG, HEX, FLU, FAM, DABSYL, Cy7, Cy5, Cy3, BOFL, BOF, BO-X, BO-TRX, BO-TMR, JOE, 6JOE, VIC, 6FAM, LCRed640, LCRed705, TAMRA, Biotin, Digoxigenin, DuO-family, and Daq-family.

Further, the present kit comprises one or more reagents/materials for use in establishing the variant alleles of the MBL gene.

Primers

The present kit comprises in one aspect primers selected from SEQ ID NO.:1, SEQ ID NO.:3, SEQ ID NO.:9, SEQ ID NO.:11, SEQ ID NO.:12, SEQ ID NO.:15, SEQ ID NO.:17, SEQ ID NO.:18, or variants thereof, or primers selected from the complementary strings of the above sequences.

In a second aspect the primer of the kit is selected from SEQ ID NO.:2, SEQ ID NO.:4, SEQ ID NO.:5, SEQ ID NO.:6, SEQ ID NO.:7, SEQ ID NO.:8, SEQ ID NO.:10, SEQ ID NO.:13, SEQ ID NO.:14, SEQ ID NO.:16, SEQ ID NO.:19, or variants thereof, or primers selected from the complementary strings of the above sequences.

The above primers have the '3 position in the polymorphic base of the sequences or their complementary strands.

In a third aspect the primer is operably linked to at least one label, such as operably linked to two different labels.

The term "operably linked" in the present context means that the primers can be linked to at least one label without the primer being impaired.

According to the invention the label may be selected from, but is not limited to TEX, TET, TAM, ROX, R6G, ORG, HEX, FLU, FAM, DABSYL, Cy7, Cy5, Cy3, BOFL, BOF, BO-X, BO-TRX, BO-TMR, JOE, 6JOE, VIC, 6FAM, LCRed640, LCRed705, TAMRA, Biotin, Digoxigenin, DuO-family, and Daq-family.

In one embodiment the primer is operably linked to a surface, such as the surface is the surface of microbeads or a DNA chip or plastic.

Identification of an allele as having implication for risk of sepsis

It is further contemplated that the present invention provides a method for identifying an individual as having an increased likelihood of responding positively to a sepsis treatment, comprising determining the allele genotype correlated with an increased likelihood of positive response to treatment, whereby the presence of the genotype identifies the subject as having an increased likelihood of responding to sepsis treatment.

The treatment mentioned herein may be any sepsis treatment, such as conventional sepsis treatment, for example antibodies.

Protein Products of the Gene(s)

Gene products of the MBL gene or peptide fragments thereof can be prepared for a variety of uses. For example, such gene products, or peptide fragments thereof, can be used for the generation of antibodies in prognostic assays.

Use as a pharmaceutical

It is within the scope of the invention to provide a pharmaceutical preparation to be used in the prevention and/or treatment of sepsis. Accordingly, the invention discloses the use of MBL for the manufacture of a medicament for the prevention and/or treatment of sepsis, severe sepsis, or septic shock.

The pharmaceutical of the invention comprises an effective amount of MBL in combination with pharmaceutically acceptable additives. Such pharmaceutical may suitably be formulated for oral, percutaneous, intramuscular, intravenous, intracranial, intrathecal, intracerebroventricular, intranasal or pulmonal administration.

Formulation

Strategies in formulation development of pharmaceuticals (medicaments and compositions) based on MBL generally correspond to formulation strategies for any other protein-based drug product. Potential problems and the guidance required to overcome these problems are dealt with in several textbooks, e.g. "Therapeutic Peptides and Protein Formulation. Processing and Delivery Systems", Ed. A.K. Banga, Technomic Publishing AG, Basel, 1995.

Injectables are usually prepared either as liquid solutions or suspensions, solid forms suitable for solution in, or suspension in, liquid prior to injection. The preparation may also be emulsified. The active ingredient is often mixed with excipients, which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are for example water, saline, dextrose, glycerol, ethanol or the like, and combinations thereof. In addition, if desired, the preparation may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH-buffering agents, which enhance the effectiveness or transportation of the preparation.

Formulations of the compounds of the invention can be prepared by techniques known to the person skilled in the art. The formulations may contain pharmaceutically acceptable carriers and excipients including microspheres, liposomes, microcapsules, nanoparticles or the like.

Administration

In a most preferred embodiment of the invention the application of an MBL based pharmaceutical is intravenous.

In a less preferred embodiment the application is subcutaneous and/or intramuscular.

In one aspect a localised or substantially localised application may be performed. The preparation may suitably be administered by injection, optionally at the site, where the active ingredient is to exert its effect.

Additional formulations which are suitable for other modes of administration include suppositories, nasal, pulmonal and, in some cases, oral formulations. For suppositories, traditional binders and carriers include polyalkylene glycols or triglycerides. Such suppositories may be formed from mixtures containing the active ingredient(s) in the range of from 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and generally contain 10-95% of the active ingredient(s), preferably 25-70%.

Other possible formulations are such suitable for nasal and pulmonal administration, e.g. inhalators and aerosols.

The active compound may be formulated as neutral or salt forms. Pharmaceutically acceptable salts include acid addition salts (formed with the free amino groups of the peptide compound) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic acid, oxalic acid, tartaric acid, mandelic acid, and the like. Salts formed with the free carboxyl group may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The preparations are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective. The quantity to be administered depends on the subject to be treated, including, e.g. the weight and age of the subject, the disease to be treated and the stage of disease. Suitable dosage ranges are of the order 1 mg to 10 mg active ingredient per administration with a preferred range of from about 3 mg to 7 mg. Administration may be performed once or may be followed by subsequent administrations. The dosage will also depend on the route of administration and will vary with the age and weight of the subject to be treated. A preferred dose would be in the interval 1.0 mg to 10 mg per 70 kg body weight.

In one embodiment it may be advantageous to administer MBL according to the invention with other substances to obtain a synergistic effect. Examples of such other substances may be a growth factor, which can induce cell differentiation, or a hormone, or a transplant of cells, including a transplant of stem cells, or gene therapy, or immuno-therapy.

In many instances it is necessary to administrate the formulation multiple times. Administration may be a continuous infusion, such as intra-ventricular infusion or administration in more doses, such as more times a day, daily, more times a week, or weekly.

It is preferred that administration of the medicament is initiated before or shortly after the individual has been subjected to the factor(s) that may lead to sepsis. For example the medicament may be administered to an individual prior to an operation to prevent said individual to develop sepsis following the operation.

Treatment

In a further aspect the invention relates to a method of treating an individual suffering from sepsis, septic shock or severe sepsis by administering a pharmaceutical composition comprising MBL to said individual.

In yet another embodiment the invention concerns a method of treating an individual suffering from sepsis, severe sepsis, or septic shock by administering to said individual a pharmaceutical composition comprising MBL to said individual.

All patent and non-patent references cited in the application are hereby incorporated by reference in their entirety.

Table 1

Primer and interpretation table for MBL PCR-SSP.

rxn no.	name	primers (5′ □ 3′) upper: 5′-primer lower: 3′-primer	PCR product size (bp)	haplotypes / interpretation table						
				LXPA	*LYPA*	*LYQA*	*HYPA*	*LYPB*	*LYQC*	*HYPD*
1	A non-B	AGTCGACCCAGATTG TAGGACAGAG CCTTTTCTCCCTTGGT GC	277	x	x	x	x	x	w	x
2	B	GCAAAGATGGGCGTG ATGA GGGCTGGCAAGACAA CTATTA	224					x		
3	A non-C	AGTCGACCCAGATTG TAGGACAGAG CCTGGTTCCCCCTTTT CTC	287	x	x	x	x	x		x
4	C	AGTCGACCCAGATTG TAGGACAGAG ACCTGGTTCCCCCTTT TCTT	288						x	
5	A non-D	AGTCGACCCAGATTG TAGGACAGAG TCCCTTGGTGCCATC ACG	270	x	x	x	x	x	x	
6	D	AGTCGACCCAGATTG TAGGACAGAG CTCCCTTGGTGCCAT CACA	271							x
7	X	CATTTGTTCTCACTGC	285	x						

		CACC CTCAGGGAAGGTTAA TCTCAG								
8	Y	CATTTGTTCTCACTGC CACG CTCAGGGAAGGTTAA TCTCAG	285		x	x	x	x	x	x
9	H	GGCTTAGACCTATGG GGCTA GCTTCCCCTTGGTGTT TTAC	272				x			x
10	L	GGCTTAGACCTATGG GGCTA GCTTCCCCTTGGTGTT TTAG	272	x	x	x		x	x	
11	P	TAGGACAGAGGGCAT GCTC AGGATCCAGGCAGTT TCCTCTGGAAGG	334	x	x		x	x		x
12	Q	TAGGACAGAGGGCAT GCTT AGGATCCAGGCAGTT TCCTCTGGAAGG	334			x			x	
	MBL exon4	GAGTTTCACCCACTTT TTCACA GCCTGAGTGATATGA CCCTTC	421							

w : weak signal

LYPB/LYQC heterozygotes give a weak signal in rxn #1

LYPB/HYPD heterozygotes give a weak signal in rxn #5

reactions #1 and #2, #3 and #4, etc. are complementary

Table 2

Clinical diagnoses at admission to the ICU in 272 consecutive patients with systemic inflammatory response syndrome (SIRS) and number of positive culture isolates

Diagnoses	a Non-operative Medical N (%)	b Positive isolates N (% of a)	c Post-operative Surgical N (%)	d Positive isolates N (% of c)
A. 4 Congestive heart failure	6 (3.4)	(0.0)		
B. 15 Pneumonia	69 (38.8)	53 (76.8)		
B. 16 COPD acute deterioration	27 (15.2)	8 (29.6)		
Epiglottitis	7 (3.9)	2 (28.6)		
L. 55 Respiratory infection			39 (41.5)	19 (48.7)
C. 21 Hepatic failure	3 (1.7)	2 (66.7)		
C. 24 G-I inflammatory disease	4 (2.2)	3 (75.0)		
M.59 G-I perforation/rupture/peritonitis			29 (30.9)	7 (24.1)
D. 30 Stroke	3 (1.7)	2 (66.7)		
D. 31 CNS infection	7 (3.9)	4 (57.1)		
D. 33 Neuromuscular	3 (1.7)	3 (100.0)		
D. 34 Seizure (status)	5 (2.8)	4 (80.0)		
E. 37 Urosepsis	12 (6.7)	11 (91.7)		
F. 39 Trauma	6 (3.4)	1 (16.7)		
O.74 Trauma			20 (21.3)	5 (25.0)
G. 41 Ketoacidosis	8 (4.5)	5 (62.5)		
G. 42 Major intoxication	13 (7.3)	5 (38.5)		
Other	5 (2.8)	2 (40.0)	6 (6.4)	(0.0)
	178 (100.0)	105 (60.0)	94 (100.0)	31 (33.0)

Non-operative medical (including patients with no surgery latest 7 days) and postoperative surgical diagnoses (surgery within last 7 days) at admittance according to the APACHE III diagnosis list for critically ill hospitalized adults [30]. COPD = chronic obstructive lung disease, G-I = gastrointestinal, CNS = central nervous system.

Table 3

MBL genotypes in healthy controls versus all patients admitted to an intensive care unit with systemic inflammatory response syndrome (SIRS)

MBL genotypes, structural alleles (MBL promoter alleles included)

Geno-types	Controls	All Patients	Geno-types	Controls	All Patients
	N %	N %		N %	N %
			YA/YA	72 (28.8)	83 (30.5)
			YA/XA	73 (29.2)	65 (23.9)
			XA/XA	12 (4.8)	3 (1.1)
Sum *A/A*	157 (62.8)	151 (55.5)	Sum *A/A*	157 (62.8)	151 (55.5)
A/B	48 (19.2)	59 (21.7)			
A/C	13 (5.2)	8 (2.9)	*YA/O*	53 (21.2)	82 (30.1)
A/D	25 (10.0)	40 (14.7)	*XA/O*	33 (13.2)	25 (9.2)
Sum *A/O*	86 (34.4)	107 (39.3)	Sum *A/O*	86 (34.4)	107 (39.3)
B/B	3 (1.2)	5 (1.8)			
B/D	3 (1.2)	7 (2.6)			
D/D	1 (0.4)	2 (0.7)			
Sum *O/O*	7 (2.8)	14(5.1)	Sum *O/O*	7 (2.8)	14 (5.1)
Total	250	272		250 (100.0)	272 (100.0)

	(100.0)	(100.0)			

A indicates normal structural allele. *O* is *Y* and *X* indicate base exchanges in codon -221, the common designation for variant alleles which profoundly influence the expression of MBL. (*B*, codon 54, *C*, codon 57 and *D*, codon 52). *X* is present only on *A* chromosomes, Controls vs patients (*A/A* vs *A/O* and *O/O*), **Chi-square for linear trend = 0.51, P=0.47 *Chi-square for linear trend =3.67, P=0.06.

Table 4

Table 4a. MBL genotypes, structural alleles, comparisons of patients without sepsis with sepsis, severe sepsis and septic shock in patients with inflammatory response syndrome (SIRS)

Alle-les, genot ype	Patients without sepsis N %	Patients with sepsis N %	Patients with severe sepsis N %	Patients with septic shock N %	Risk ratio sepsis* (95%CI)	Risk ratio severe sepsis* (95%CI)	Risk ratio septic shock* (95%CI)
Sum *A/A*	55 (73.3)	96 (48.7)	83 (48.5)	31 (44.3)	1	1	1
A/B	*9 (12.0)*	*50 (25.4)*	*45 (26.3)*	*14 (20.0)*			
A/C	*2 (2.7)*	*6 (3.1)*	*5 (2.9)*	*3 (4.3)*			
A/D	*9 (12.0)*	*31 (15.7)*	*26 (15.2)*	*13 (18.6)*			
Sum *A/O*	20 (26.7)	87 (44.2)	76 (44.4)	30 (42.9)	1.28 (1.1-1.49)	1.32 (1.11-1.56)	1.66 (1.16-2.39)
B/B	*0*	*5 (2.5)*	*4 (2.3)*	*3 (4.3)*			
B/D	*0*	*7 (3.6)*	*6 (3.5)*	*4 (5.7)*			
D/D	*0*	*2*	*2*	*2*			

		(1.0)	*(1.2)*	*(2.9)*			
Sum *O/O*	0	14 (7.1)	12 (7.0)	9 (12.9)	1.57 (1.39-1.77)	1.66 (1.45-1.9)	2.77 (2.09-3.68)
Total	75 (100.0)	197 (100.0)	171 (100.0)	70 (100.0)			

Table 4b.

MBL promoter alleles included

	Patients without sepsis N %	Patients with sepsis N %	Patients with severe sepsis N %	Patients with septic shock N %	Risk ratio with sepsis** (95%CI)	Risk ratio with severe sepsis** (95%CI)	Risk ratio with septic shock** (95%CI)
YA/YA	31 (41.3)	52 (26.4)	45 (26.3)	14 (20.0)	1	1	1
YA/XA	24 (32.6)	41 (20.8)	36 (21.1)	17 (24.3)	1.01 (0.78-1.29)	1.01 (0.77-1.34)	1.33 (0.76-2.35)
XA/XA	0	3 (1.5)	2 (1.2)	0	1.60 (1.35-1.88)	1.69 (1.40-2.04)	N/A
YA/O	17 (22.7)	65 (33.0)	55 (32.2)	21 (30.0)	1.27 (1.04-1.54)	1.29 (1.03-1.62)	1.78 (1.06-2.99)
XA/O	3 (4.0)	22 (11.2)	21 (12.3)	9 (12.9)	1.40 (1.13-1.75)	1.48 (1.16-1.88)	2.41 (1.40-4.15)
O/O	0	14 (7.1)	12 (7.0)	9 (12.9)	1.60 (1.35-1.88)	1.69 (1.40-2.04)	3.21 (2.08-4.96)

*(*A/A* vs *A/O* and *O/O*) Chi-square for linear trend < 15.1, P <0.001. **Chi-square for linear trend < 15.8, P<0.001.

Table 5

Microbial species diagnosed in cultures taken at admission (day -3 to +1) to intensive care unit in 272 consecutive patients with systemic inflammatory response syndrome (SIRS).

	Total (%)	***A/A* (%)**	***A/O* (%)**	***O/O* (%)**
Patients				
Sum positive	**136 (50.0)**	**68 (45.0) ***	**56 (52.3) ***	**12 (85.7) ***
Sum negative or unknown	136 (50.0)	83 (55.0) *	51 (47.7) *	2 (14.3) *
Total	272	151	107	14
Microorganisms				
Gram positive	**65 (47.7)**	**34 (50.0)**	**25 (44.6)**	**6 (50.0)**
Staphylococcus aureus	21 (32.3)	11 (32.4)	9 (36.0)	1 (16.7)
Streptococcus pneumon.	17 (26.2)	8 (23.5)	6 (24.0)	3 (50.0)
Staphylococcus albus	15 (23.0)	10 (29.4)	5 (20.0)	0
Streptococcus faecalis	6 (9.2)	1 (2,9)	4 (16.0)	1 (16.7)
Streptococcus pyogen. A	2 (3.1)	2 (5.9)	0	0
Other Gram positive	4 (6.2)	2 (5.9)	1 (4.0)	1 (16.7)
Gram negative	**57 (41.9)**	**27 (39.7)**	**25 (44.6)**	**5 (41.7)**
Escherichia coli	24 (42.1)	10 (37.0)	10 (40.0)	4 (80.0)
Haemophilus influenzae	13 (22.8)	6 (22.2)	7 (28.0)	0
Klebsiella pneumoniae	5 (8.8)	1 (3.7)	4 (16.0)	0
Pseudom. aeruginosae	3 (5.3)	3 (11.1)	0	0
Serratia marcescens	3 (5.3)	3 (11.1)	0	0
Branhamella	3 (5.3)	2 (7.4)	1 (4.0)	0
Other Gram negative	6(10.6)	2 (7.4)	3 (12.0)	1 (20.0)
Fungi				
Candida albicans	14 (10.3)	7 (10.3)	6 (10.7)	1 (8.3)

173 cultures were found positive in 136 patients (40 from blood, 97 from trachea, 26 from urine and 10 from other sources). A species appearing in more cultures per patient is only counted once.

A indicates the normal structural allele. *O* is the common designation for variant alleles (*B*, codon 54, *C*, codon 57 and *D*, codon 52) *Chi square for linear trend = 6.02, P = 0.012.

Table 6

MBL genotypes in patients with systemic inflammatory response syndrome (SIRS). Comparison between survivors and non-survivors during stay at hospital

Table 6a. MBL genotypes, structural alleles

Genotypes	Survivors N %	Non-survivors N %	Risk ratio (95%CI)
Sum *A/A*	112 (59.3)	39 (47.0)	1
A/B	*38 (20.1)*	*21 (25.3)*	
A/C	*5 (2.6)*	*3 (3.6)*	
A/D	*27 (14.3)*	*13 (157.)*	
Sum *A/O*	70 (37.0)	37 (44.6)	1.34 (0.92-1.95)
B/B	*4 (2.1)*	*1 (1.2)*	
B/D	*1 (0.5)*	*6 (7.2)*	
D/D	*2 (1.1)*	*0 (0.0)*	
Sum *O/O*	7 (3.7)	7 (8.4)	1.94 (1.07-3.49)
Total	189 (100.0)	83 (100.0)	

Chi-square for linear trend = 4.7, P=0.030

Table 6b. MBL promoter alleles included

Genotypes	Survivors N %	Non-survivors N %	Risk ratio (95%CI)
YA/YA	65 (34.4)	18 (21.7)	1
YA/XA	44 (23.3)	21 (25.3)	1.49 (0.87-2.55)
XA/XA	3 (1.6)	0	N/A
YA/O	53 (28.0)	29 (34.9)	1.63 (1.0-2.70)
XA/O	17 (9.0)	8 (9.6)	1.48 (0.7-2.98)

O/O	7 (3.7)	7 (8.4)	2.31 (1.19-4.48)
Total	189 (100.0)	83 (100.0)	

Chi-square for linear trend =4.7, P=0.030

Table 7

Simplified Acute Physiology Score (SAPS II) parameters, observed and predicted mortality in 272 patients with systemic inflammatory response syndrome (SIRS) classified by mannose-binding lectin structural variant alleles.

	Total	A/A	A/O	O/O
Patients	272	151	107	14
Age				
Age (y)	60.2 (±16.5)	60.1 (±16.7)	60.7 (±16.3)	55.5 (±17.8)
Type of admission				
Acute surgery	86 (31.6%)	51 (33.8%)	33 (30.8%)	2 (14.3%)
Elective surgery	8 (2.9%)	5 (3.3%)	3 (2.8%)	0
Medical*	178 (65.4%)	95 (62.9%)	71 (66.4%)	12 (85.7%)
Chronic disease**				
AIDS	0	0	0	0
Metastatic cancer	1	1	0	0
Hematological malignancy	3	3	0	0
SAPS II				
SAPS II score	37.7 (±15.4)	36.8 (±15.1)	38.4 (±16.3)	42.1 (±10.6)
Age points	10.4 (±5.4)	10.4 (±5.2)	10.4 (±5.7)	10.1 (±5.7)
Acute physiology points ***	27.2 (±13.3)	26.4 (±12.7)	28.0 (±14.4)	30.4 (±11.2)

Mortality				
Mortality Observed	83 (30.5%)	39 (25.8%)	37 (34.6%)	7 (50.0%)
Mortality Predicted****	83.0 (30.5%)	44.6 (29.5%)	33.4 (31.2%)	5.0 (35.7%)
SMR	1.00	0.87	1.11	1.40
Number of SIRS criteria				
2	71 (26.1%)	43 (28.5%)	27 (25.2%)	1 (7.1%)
3	91 (33.5%)	51 (33.8%)	36 (33.6%)	4 (28.6%)
4	110 (40.4%)	57 (37.7%)	44 (41.1%)	9 (64.3%)

A indicates the normal structural allele. *O* is the common designation of the variant alleles (*B*, codon 54, *C*, codon 57 and *D*, codon 52). Mean values or numbers. Brackets indicate ± SD or %.

* Without surgery latest 7 days.

** Chronic disease is based on available information on day 1.

***Acute physiology score (APS), combined score for heart rate, systolic blood pressure, temperature, white blood cell count, PaO_2/FIO_2 ratio, urinary nitrogen, bilirubin, natrium, potassium, bicarbonate, diuresis and GCS (Glasgow Coma Score).

**** Predicted mortality after 1st level customization (logit=-3,8084+(0,0741*SAPS II). SMR is standardized mortality rate (mortality observed/mortality predicted). Continuous data was analysed by Kruskall-Wallis test and categorical data by Chi^2 test. None of the parameters deviated significantly between the different MBL genotypes (P>0.05).

Description of Drawings

Figure 1: MBL genotyping pattern. MBL genotyping by PCR- Sequence Specific Priming (SSP). The numbers 1-12 indicate the PCR reactions listed in table. M: Molecular weight marker, pBR327/Hae III.

Figure 2: Relationship between MBL serum concentrations and MBL genotypes. MBL serum concentrations in 272 patients with systemic inflammatory response syndrome (SIRS) are shown in relation to MBL structural alleles (*O*) as well as the MBL promoter alleles in position -221 (*X/Y*). Ranges, quartiles and medians are indicated. The detection limit in the assay is 20 µg/L.

Figure 3: Survival plot stratified according to MBL genotype. Kaplan-Meier plots are shown for patients with systemic inflammatory response syndrome (SIRS) from time of admission to the intensive care unit until discharge from hospital. Events are defined as time of death. Open circles indicate events for SIRS patients with the *A/A* genotype, closed circles the *A/O* genotype and open quadrangles the *O/O* genotype. Panel A shows the figure for patients below the 50 percentile (63.5 years of age) (log rank P< 0.001) and panel B shows the figures for patients above the 50 percentile (log rank P=0.9).

Table 1: show the primers and the MBL haplotypes.

Table 2: shows the clinical diagnosis of ICU patients with SIRS.

Table 3: shows the MBL genotypes of SIRS patients.

Table 4: shows microbiological species in cultures taken from SIRS patients.

Table 5: shows MBL genotypes in individuals with or without sepsis.

Table 6: shows MBL genotypes in individuals with or without SIRS.

Table 7: shows a SAPS II score for patients having SIRS and with MBL structural variant alleles.

Table 8: Demographic and clinical variables in survivors and non-survivors.

Examples

METHODS

Patients: from February 1998 to July 1999 all patients above 17 years of age (range: 18-88 years) admitted to an academic multidisciplinary ICU at Glostrup University Hospital in Copenhagen, Denmark and fulfilling the criteria of SIRS as outlined by Bone et al., were included in the study (n=272, 132 females and 140 males). The protocol was approved by the local ethic committee in the County of Copenhagen. Informed consent was obtained from the patients or from their relatives. In appendix 1 is outlined the SIRS and sepsis

criteria used in this study. Information about death at a follow-up status in February 2002 was obtained from the Danish Central Office of Civil Registration. As background controls served 190 blood donors and 60 members of the hospital staff.

Simplified acute physiology score II (SAPS II) AND MORTALITY PREDICTION

SAPS II is based on a large international sample of medical and surgical patients and provides an estimate of the risk of death without having to specify a diagnostic category. The parameters included are listed in table 7. The worst values within the first 24 h after admission to the intensive care unit were recorded.

DETECTION OF MBL PROTEIN CONCENTRATIONS AND GENOTYPES

MBL concentrations in serum were measured in a double enzyme immuno-assay as previously described (Garred et al. 1992). MBL single nucleotide polymorphism's (SNPs) in form of the structural variants named *B* (codon 54), *C* (codon 57), and *D* (codon 52) as well as the regulatory variants named H/L (-550), X/Y (-221), and P/Q (+4) were typed by a PCR and sequence specific primers (PCR-SSP) using the 12 reactions listed in table 1. As internal positive control a PCR covering exon 4 of the *mbl2* gene was included. The PCR was performed essentially as previously described (Aldener et al. 1996), except that the concentration of dNTPs was reduced to 0.7 mM, and the PCR products were analysed by a 2% agarose gel electrophoresis. Figure 1 shows seven patterns necessary for covering all combinations of each of the six complementary reactions. Although the typing was performed as SNP-typing the results were combined in haplotypes, based on strong linkage equilibrium between the SNPs. All three structural alleles (*B*, *C*, and *D*) have a considerable effect on MBL concentrations and to avoid small groups, the three alleles were grouped in one category (called allele "*O*") for statistical analyses. The normal allele is designated *A*. The following 6 MBL genotypes were defined: the *A/A* group: two normal structural alleles with high-expression promoter activity in position -221 (*YA/YA*) or one high-expression promoter and one low-expression promoter (*YA/XA*) or two low-expression promoters (*XA/XA*); the *A/0* group: one variant structural allele (i.e. defective allele) and one normal structural allele regulated by a high-expression promoter (*YA/0*) or a low-expression promoter (*XA/0*) and the *0/0* group with two defective structural alleles.

Statistical analyses

Contingency table analyses and trend analyses were used to compare frequencies. Kruskall-Wallis or Mann-Whiney tests were used to compare continuous data. When appropriate, logistic regression analyses were used to investigate for possible confounders. Survival analyses were performed with Kaplan-Meier plot and assessed by the log rank test. The SAPS II scores were converted into a prediction of mortality as originally

outlined. To create a current probability of mortality for the present dataset the SAPS II scores were calibrated to the actual mortality using the receiver operating characteristic curve (ROC-curve) analysis and goodness-of-fit tests as outlined in appendix 2.

RESULTS

Out of 272 patients enrolled with SIRS a total of 197 (72.4%) fulfilled the sepsis criteria either at admission or during the first 24 h in the ICU (table 2). The frequency of MBL genotypes did not deviate significantly between patients with SIRS and healthy controls (table 3). The distribution of the MBL serum concentrations was closely associated with the different MBL genotypes ($P<0.001$) (figure 2). 50.0% of the patients were culture positive in specimens taken immediately before or at admission to the ICU (table 4). The microbiological spectrum was wide and no infectious agent was predominant (table 4). However, a significant increased proportion of the carriers of MBL variant alleles were culture positive in a gene dose dependent manner ($P=0.012$) (table 4). Among the 197 patients with sepsis, 171 (86.8%) were classified as having severe sepsis. Furthermore, 75 (43.8%) of these patients were classified as having septic shock (table 5). Stratification of the patients according to MBL genotypes revealed that a highly significant proportion of the patients with sepsis carried MBL variant alleles compared with the patients not meeting the sepsis criteria ($P<0.001$) (table 5). Further analyses showed that carriers of MBL variant alleles also had a high risk of development of severe sepsis and septic shock ($P<0.001$) (table 5). When the promoter alleles were taken into account there was a striking linear trend in susceptibility for sepsis, severe sepsis and development of septic shock from the highest expressing MBL genotypes to genotypes encoding MBL deficiency when compared with SIRS patients without sepsis ($P<0.001$) (table 5). Moreover, an independent and significant association with the presence of MBL variant alleles (*A/A* versus *A/O+O/O*) and sepsis ($n=26$) was observed when the patients with severe sepsis and septic shock were excluded from the analysis (chi-square: 4.77, 1 DF, $P=0.028$); and also when patients classified as having septic shock were excluded from the analysis of the severe sepsis group ($n=101$) (chi-square: 5.25, 1 DF, $P=0.022$). When the MBL gene dose dependent risk of having sepsis versus healthy controls was compared the same tendency was observed, although not as prominent (table 3 and table 5) (chi-square for linear trend= 10.8, $P=0.001$). Age was a significant risk factor for developing sepsis (logistic regression, $P=0.03$). However, the risk of developing sepsis in patients carrying MBL variant alleles was independent of age when tested in a logistic regression analysis ($P<0.001$).

In total, 83 (30.5%) of the patients died during admission to hospital (table 6). Among these were an increased gene dose dependent fraction of patients who carried MBL variant alleles, i.e. 25.8% of those with the normal genotype, 34.6% of the heterozygous, and

50.0% of the homozygous defective gene died during the in-hospital period (Chi-square for linear trend = 4.7, P=0.030). Inclusion of the MBL promoter alleles suggested that those with the highest MBL levels (*YA/YA*) were most protected against fatal outcome (chi-square for linear trend =4.7, P=0.030) (table 6). It should be noted that an increased risk of fatal outcome for MBL variant allele carriers was present in both the sepsis as well as the non-sepsis group, but neither was significant at the 5% level. Also a reduced MBL serum concentration was observed in the non-survivor group compared with the survivor group (mean 997 µg/L ± SD 1225 and 1398 µg/L ± SD 1456, respectively, P=0.020). Older age was a very strong predictor of poor outcome in this study (logistic regression P<0.001). Thus we investigated whether the association between MBL and poor outcome was related to the younger patients, by using an arbitrarily limit at the 50% percentile (n=136, 63.5 years of age). As can be seen in figure 3, the death rate is twice as high in the older patients but the association of MBL variant alleles with fatal outcome in general and with homozygosity in particular is predominant in the younger patients (log rank P<0.001). However, no association was seen in the older patients (log rank P=0.9). In the follow up period additional 68 patients died (mean follow up time: 30.3 months). No significant overall association was seen between MBL variant alleles and death in this period (chi-square for trend = 0.22, P=0.64).

No significant differences in the parameters underlying the SAPS II score, the crude SAPS II score and the predicted standardized mortality rate based on the SAPS II score were observed when stratified according to MBL genotypes (P>0.05) (table 7). Neither was there any significant association with increasing numbers of SIRS criteria (2 to 4) and the MBL genotypes (table 7).

Characteristics between survivors and non-survivors are listed in table 8. The SAPS II score and the number of SIRS criteria are clearly associated with fatal outcome. However, no significant association between SAPS II score, number of SIRS criteria and MBL genotypes was found.

DISCUSSION

Many factors are involved in controlling and limiting localized infections. In general, the septic response occurs when immune defences fail to contain an invading microbe. Many cases of sepsis are triggered by microbes that do not ordinarily cause systemic disease. Deficiencies in non adaptive innate host factors have been suggested to be of particular importance, but so far the epidemiological proof of such a notion has been limited. This study shows that decreased levels and lack of functional MBL is crucial in order to avoid development of sepsis and septic shock in critically ill patients. Patients with SIRS have a

high risk of developing sepsis provided they carry variant alleles in the *mbl2* gene, which decrease the level of MBL in the blood. Although not as prominent the same difference was observed when sepsis patients were compared with healthy controls. Thus, MBL seems to be crucial in controlling systemic dissemination of different infectious agents in patients with acute medical and surgical stress. This observation is also in agreement with the finding that a significant increased proportion of patients carrying MBL variant alleles also had a positive bacterial blood culture.

In general, MBL exert its largest effect during the vulnerable window of infancy between 6 and 18 months of age. Nevertheless, MBL deficiency has been associated with a number of infections, particular in patients with concomitant immunodeficiencies. The initial SIRS insult creates a precondition rendering the patient partly immunocompromised. This increases the patient's susceptibility for infection, thereby exposing the clinical MBL phenotype. The necessity that an accompanying condition has to be present before MBL deficiency becomes clinically important has recently been indicated in relation to pneumococcal pneumonia. In unselected patients a variable association is seen, while in selected patients with a concomitant disorder a clear association is revealed.

The finding that the frequency of MBL variant alleles increases with the severity of sepsis (severe sepsis and septic shock) indicates that lack of the buffering capacity of MBL towards initial microbial replication not only is associated with susceptibility for infection but also allows activation of host mechanisms central to the pathophysiology of the sepsis syndrome. In agreement with this view are several *in vitro* findings indicating that MBL may suppress the release of proinflammatory cytokines. Thus, MBL may have both a direct antimicrobial role and it may also have a modulating effect on the inflammatory response. Recently, MBL has been shown to function as scavenger molecule towards cells undergoing apoptosis and necrosis. Lack of functional MBL could also very well play a role in the development of sepsis severity.

During hospital stay an increased risk of fatal outcome in the patients carrying MBL variant alleles were observed. By contrast no such association was observed in the 30 months follow-up period indicating that the increased risk of death was directly related to the actual pathological incidence. Another important aspect seems to be older age, which is a strong risk factor for fatal outcome in patients with sepsis (Bone et al. 1992). When the oldest patients (above median) were excluded from the analyses it was revealed that the MBL variant alleles were solely associated with fatal outcome in the younger patients. This indicates that multiple additive factors others than inherited factors may be important for prognosis in the older patients. However, the close association between MBL deficiency and the development of sepsis, which was higher than the association with fatal outcome suggests that MBL may influence processes related to the initial steps in the disease

process. Compatible with this view is the lack of association between MBL variant alleles and the SAPS II score and between MBL variant alleles and an increasing number of SIRS criteria, which is also clearly associated with the risk of fatal outcome (Bone et al. 1992). In this regard it is interesting that the excessive activation of complement that takes place in SIRS and sepsis has been shown to be associated with fatal outcome. Moreover, genetic complement deficiency or complement depletion has been shown to be beneficial in animal models of complement-dependent inflammation. Thus, complement and probably MBL may play different roles during the path of the sepsis syndrome, which may explain why it is difficult to classify SIRS patients with aberrations in their MBL genetic profile in relation to prognosis.

To conclude, this study shows that genetically determined differences in the MBL gene (*mbl2*) explain a significant proportion of an inherited risk of developing sepsis in critically ill patients. MBL variant alleles were also associated with increased risk of death. Accordingly, rapid determination of the MBL genotype is important to identify patients at risk for developing severe sepsis. Moreover, since MBL substitution therapy now is possible our data raise the prospective that MBL may be used in prophylaxis and treatment of the sepsis syndrome.

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Claims

1. A method of predicting whether an individual having Systemic Inflammatory Response Syndrome (SIRS) will develop sepsis comprising the steps of:

a) providing a biological sample from said individual,

b) determining the mannose-binding lectin (MBL) genotype of said individual,

c) predicting the risk of said individual of developing sepsis by correlating the MBL genotype determined in step b) with a predefined risk value associated with said particular MBL genotype.

2. The method according to claim 1, wherein the MBL genotype has at least one variant structural allele of the MBL gene.

3. The method according to claim 2, wherein the variant structural allele has in codon 52 in position 1045 a base T (SEQ ID NO: 25 (GeneBank, ID No. Y16582)) and/or in codon 54 in position 1052 has a base A (SEQ ID NO: 23 (GeneBank, ID No. Y16579)) and/or in codon 57 in position 1055 has a base A (SEQ ID NO: 22 (GeneBank, ID No. Y16576)).

4. The method according to any of the preceding claims, wherein the MBL has two low-expression regulatory alleles of the MBL gene.

5. The method according to claim 4, wherein the low-expression regulatory allele has in position 602 a base C substitution (SEQ ID NO: 26 (GeneBank, ID No. Y16580).

6. The method according to any of the preceding claims, wherein the at least one structural allele or the at least one regulatory allele is subjected to amplification.

7. The method according to any of the preceding claims, wherein the amplification is performed by an amplification method selected from the group consisting of at least one of polymerase chain reaction (PCR), Ligase Chain Reaction (LCR), Nucleic Acid Sequence-Based Amplification (NASBA), strand displacement amplification, rolling circle amplification, and T7-polymerase amplification.

8. The method according to any of the preceding claims, wherein the presence of the at least one allele is determined by hybridising a primer to a target nucleic acid sequence

comprising at least one structural allele or at least one regulatory allele, or hybridising to any complementary sequence of said alleles.

9. The method according to any of the preceding claims, wherein the amplification is carried out by means of sequence-specific primers (SSP).

10. The method according to any of the preceding claims, wherein the amplification employs the following sequence-specific primer pairs selected form the group consisting of at least one of:

5' primer: AGTCGACCCAGATTGTAGGACAGAG (SEQ ID NO:1)
3' primer: CCTTTTCTCCCTTGGTGC (SEQ ID NO:2),

5' primer: GCAAAGATGGGCGTGATGA (SEQ ID NO:3)
3' primer: GGGCTGGCAAGACAACTATTA (SEQ ID NO:4),

5' primer: AGTCGACCCAGATTGTAGGACAGAG (SEQ ID NO:1)
3' primer: CCTGGTTCCCCCTTTTCTC (SEQ ID NO:5),

5' primer: AGTCGACCCAGATTGTAGGACAGAG (SEQ ID NO:1)
3' primer: ACCTGGTTCCCCCTTTTCTT (SEQ ID NO:6),

5' primer: AGTCGACCCAGATTGTAGGACAGAG (SEQ ID NO:1)
3' primer: TCCCTTGGTGCCATCACG (SEQ ID NO:7),

5' primer: AGTCGACCCAGATTGTAGGACAGAG (SEQ ID NO:1)
3' primer:CTCCCTTGGTGCCATCACA (SEQ ID NO:8),

5' primer: CATTTGTTCTCACTGCCACC(SEQ ID NO:9)
3' primer: CTCAGGGAAGGTTAATCTCAG (SEQ ID NO:10),

5' primer: CATTTGTTCTCACTGCCACG (SEQ ID NO:11)
3' primer: CTCAGGGAAGGTTAATCTCAG (SEQ ID NO:10),

5' primer: GAGTTTCACCCACTTTTTCACA (SEQ ID NO:18)
3' primer: GCCTGAGTGATATGACCCTTC (SEQ ID NO:19).

11. A method of predicting whether an individual having SIRS will develop sepsis comprising the steps of:

a) providing a biological sample from said individual,

b) determining the MBL concentration in said individual,

c) predicting the risk of said individual of developing sepsis by correlating the MBL concentration of step b) with a predefined risk value associated with said particular MBL concentration.

12. The method according to claim 11, wherein the MBL concentration is determined by the means of ELISA, RIA or TRIFMA.

13. The method according to any of claims 11-12, wherein the MBL concentration is determined by determining the activity of MASP (MBL associated serine proteases).

14. A method of predicting whether an individual having SIRS will develop sepsis comprising combining the two methods according to any of the preceding claims.

15. The method according to any of the preceding claims, wherein the biological sample is provided from an individual having at least two indications of systemic inflammation response syndrome (SIRS) selected from the group consisting of

(1) a core temperature of ≥ 38°C or ≤36°C;

(2) a heart rate of >90 beats/min;

(3) a respiratory rate of ≥ 20 breaths/min, a $PaCO_2$ ratio of ≤ 4.3 kPa (32 mm Hg), or a need for mechanical ventialation;

(4) a white blood cell count of ≥ $12.0x10^9$ cells/l or ≤$4.0x10^9$ cells/l or a differential count showing > 10% immature neutrophils

16. The method according to any of the preceding claims, said individual has a white blood cell count of ≥ $10.0x10^9$ cells/l.

17. The method according to any of the preceding claims, wherein said method is carried out within 168 hours of the initiation of the indications of SIRS.

18. A kit for predicting whether an individual will develop sepsis comprising:

at least one 5' primer selected from the group comprising the nucleic acid sequences as defined in SEQ ID NO.:1, SEQ ID NO.:3, SEQ ID NO.:9, SEQ ID NO.:11, and SEQ ID NO.:18.

5

at least one 3' primer selected from the group comprising the nucleic acid sequences as defined in SEQ ID NO.:2, SEQ ID NO.:4, SEQ ID NO.:5, SEQ ID NO.:6, SEQ ID NO.:7, SEQ ID NO.:8, SEQ ID NO.:10 and SEQ ID NO.:19 for amplification of variant alleles of the MBL gene.

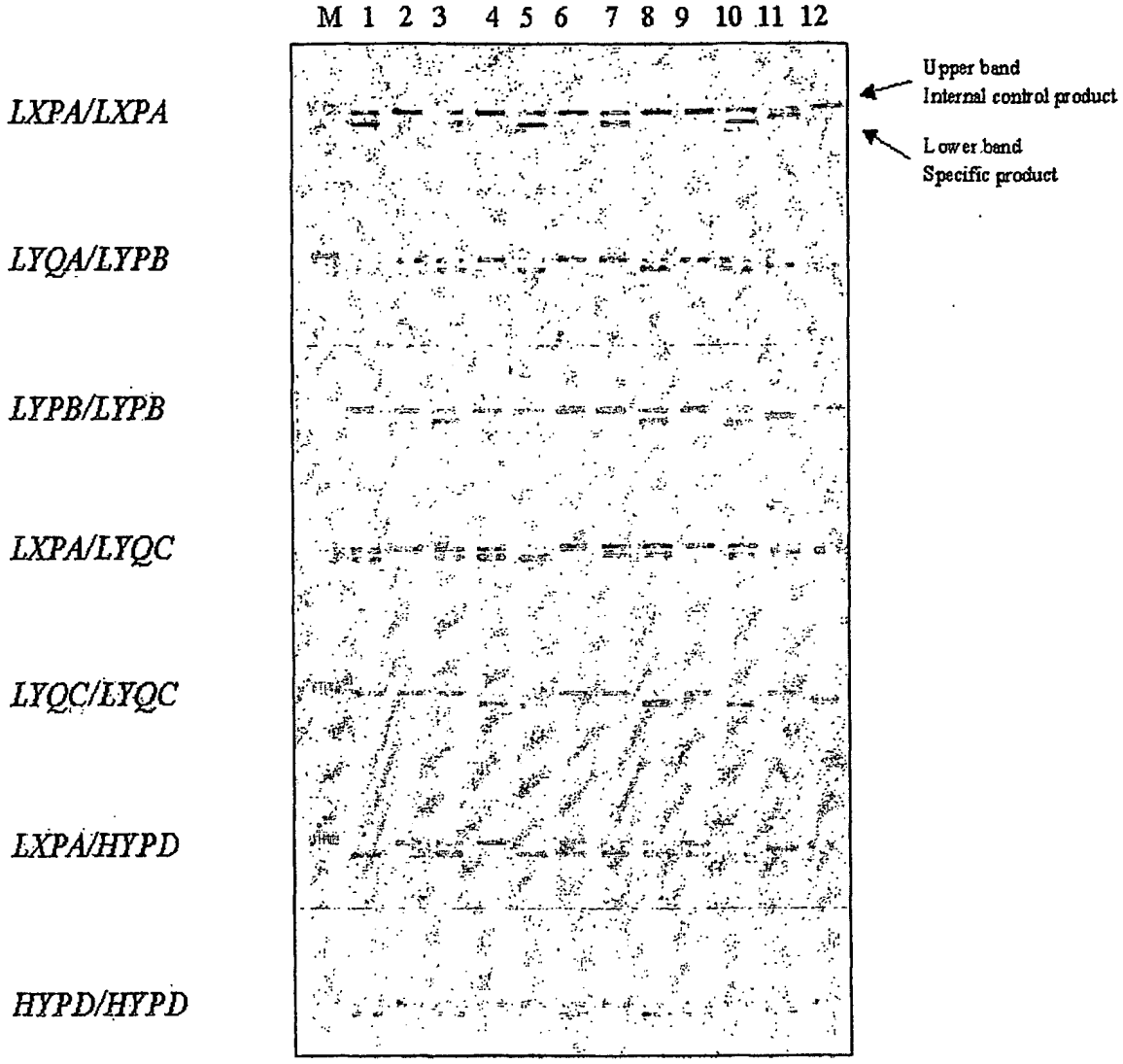
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Fig. 1

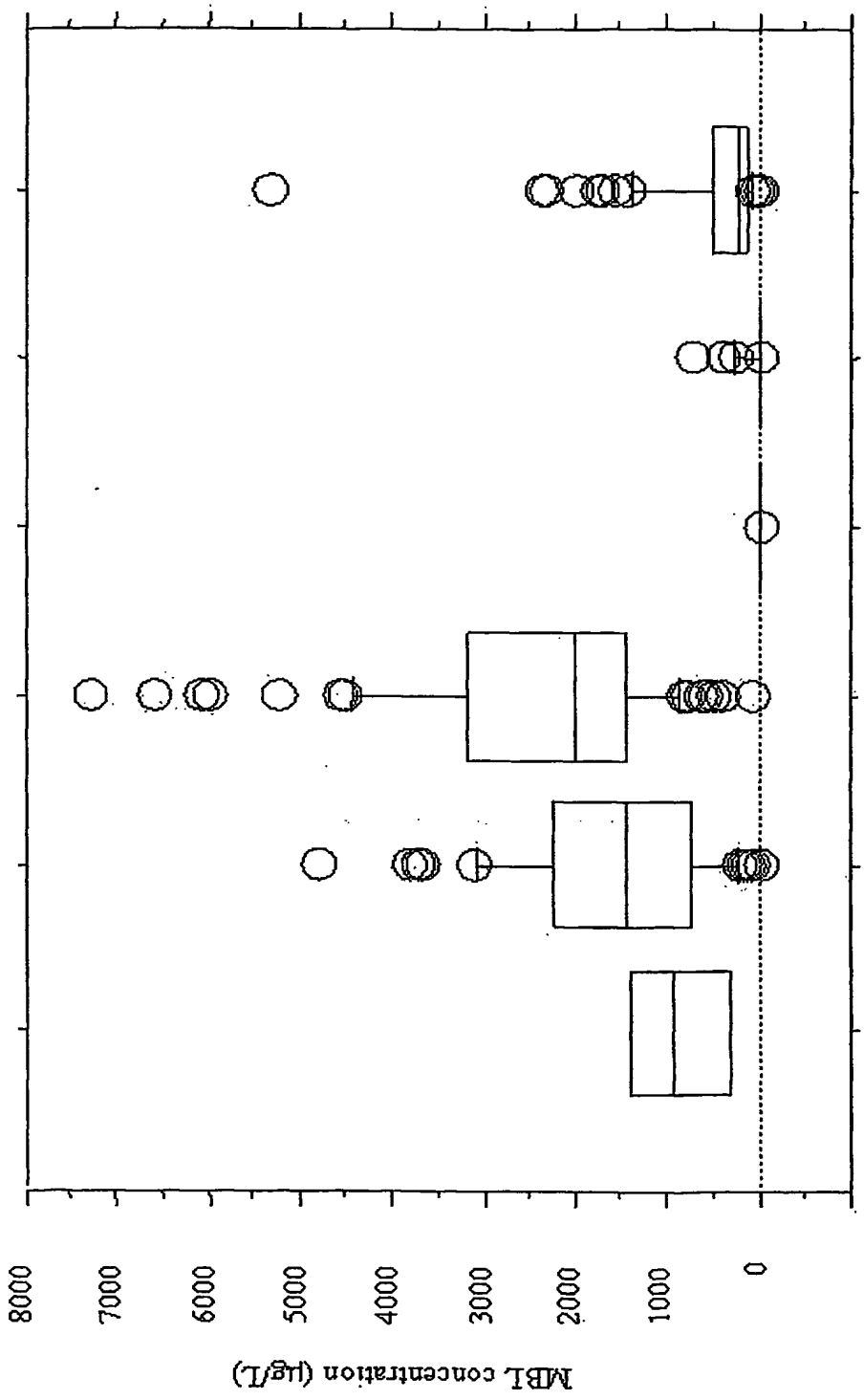
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Fig. 2

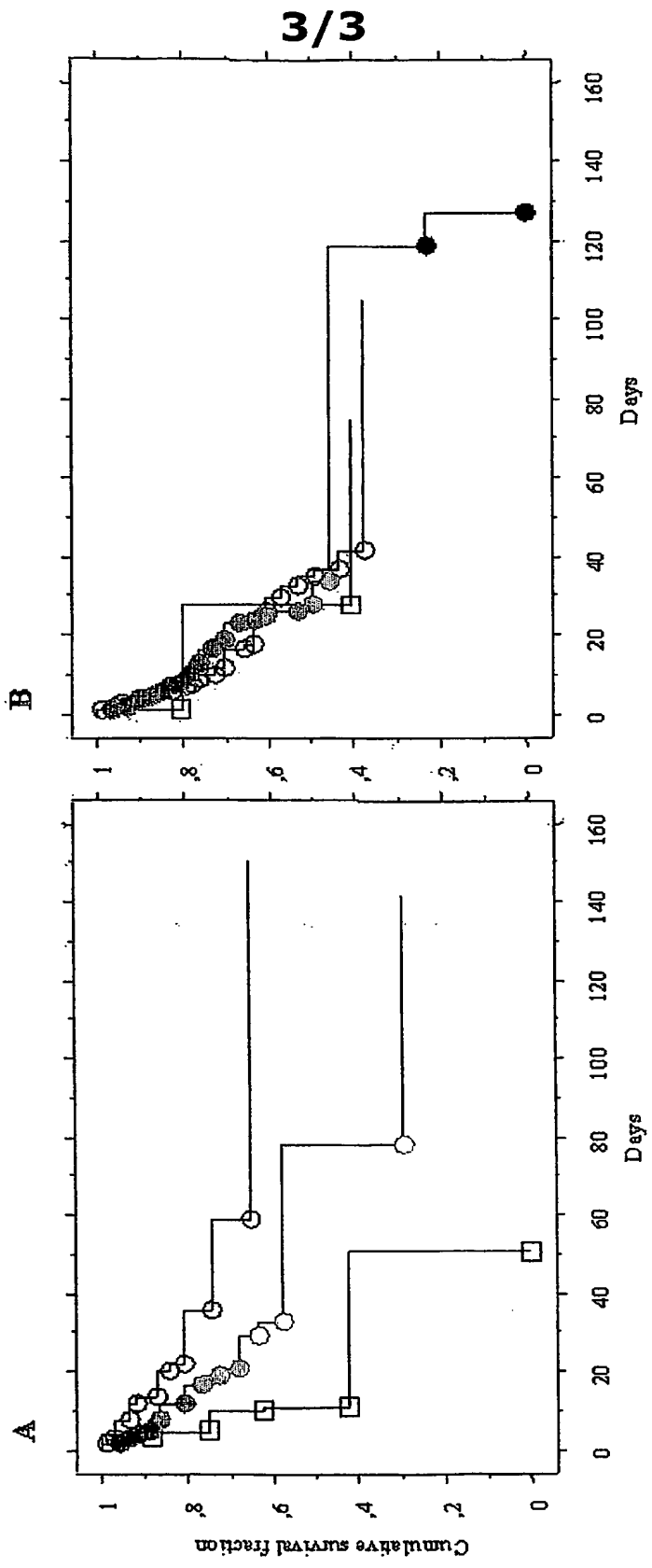
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Fig. 3

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